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A receptor-based bioadsorbent to target advanced glycation end products in chronic kidney disease

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Abstract

The accumulation of advanced glycation end products (AGEs) has been reported to be a major contributor to chronic systemic inflammation. AGEs are not efficiently removed by hemodialysis or the kidney of a chronic kidney disease (CKD) patient. The goal of this study was to develop a receptor for AGEs (RAGE)-based bioadsorbent device that was capable of removing endogenous AGEs from human blood. The extracellular domain of RAGE was immobilized onto agarose beads to generate the bioadsorbent. The efficacy of AGE removal from saline, serum, and whole blood; biological effects of AGE reduction; and hemocompatibility and stability of the bioadsorbent were investigated. The bioadsorbent bound AGE-modified bovine serum albumin (AGE-BSA) with a binding capacity of 0.73 ± 0.07 mg AGE-BSA/ml bioadsorbent. The bioadsorbent significantly reduced the concentration of total AGEs in serum isolated from end stage kidney disease (ESKD) patients by 57%. AGE removal resulted in a significant reduction of vascular cell adhesion molecule-1 (VCAM-1) expression in human endothelial cells and abolishment of osteoclast formation in osteoclast progenitor cells. A hollow fiber device loaded with bioadsorbent reduced endogenous AGEs from recirculated blood to 36% of baseline levels with no significant changes in total protein and albumin concentration. The bioadsorbent

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maintained AGE-specific binding capacity after freeze-drying and storage for 1 year. This approach provides the foundation for further development of sRAGE-based extracorporeal therapies to selectively deplete serum AGEs from human blood and decrease inflammation in patients with diabetes and/or CKD.

Keywords

AGEs; RAGE; inflammation; ESKD; extracorporeal; hollow fiber device

INTRODUCTION

Advanced glycation end products (AGEs) are a complex and heterogeneous group of compounds that are formed by non-enzymatic glycation and oxidation of proteins, lipids and nucleic acids (1). The chronic hyperglycemic state or oxidative and carbonyl stress found in diabetic and chronic kidney disease (CKD) patients, respectively, can lead to the accumulation of AGEs in serum. AGEs have been shown to be important mediators in the pathology of diabetic nephropathy, retinopathy, neuropathy, hypertension, and accelerated atherosclerosis, major causes of morbidity and mortality among patients with diabetes and end stage kidney disease (ESKD) (1). Long-term prospective studies have demonstrated that high serum AGE concentration predicted cardiovascular disease (CVD) deaths in patients with diabetes, independently of traditional CVD risk factors (2, 3). These facts are important because in the United States, there are 23.6 million people with diabetes, one of the major causes of ESKD and approximately 370,000 of the ESKD patients must undergo long-term hemodialysis (4). Furthermore, 67% of patients undergoing hemodialysis will prematurely die within five years, mainly due to cardiovascular disease (4). Therefore, therapies that decrease AGEs in these patients may have a positive impact on the incidence or type of cardiovascular events that affect them.

Accumulated AGEs contribute to tissue damage via direct chemical cross-linking of structural proteins such as collagen, or through cell surface receptor-mediated pathways (5). The receptor for AGE (RAGE) has been shown to interact with AGEs as well as the inflammatory mediators human motility group box-1 (HMGB-1), β-amyloid, and s100 calgranulins (6–8). The binding of RAGE has been shown to trigger the $p21^{ras}$/MAP kinase signaling cascade and increase the expression of NF-κB-controlled genes, including pro-inflammatory cytokines, vasoconstrictors and adhesion molecules (9).

AGEs are not efficiently removed by hemodialysis (5). Therefore, strategies aimed at normalizing AGE concentrations in the blood are being investigated. Pharmaceutical approaches based on carbonyl scavengers, inhibitors of Amadori product conversion to AGEs, and so-called “AGE crosslink breakers” (i.e., alagebrium) have been tested in clinical trials with some beneficial effects, but concerns about the safety and efficacy of these agents remain (1, 10). The repeated systemic injection of soluble RAGE has been examined for its ability to inhibit atherosclerosis and diabetic complications in mouse models (11). However, sRAGE treatment has been shown to increase cytokine production and lead to microvesicular steatosis (12). Although one group investigated the use of immobilized

lysozyme as a method to remove AGEs from blood (13), this approach is non-specific. Therefore, a different approach is warranted to specifically reduce elevated serum concentrations of AGEs without negative systemic side effects. Herein, we describe the use of the soluble extracellular domain of RAGE (sRAGE) in a bioadsorbent to target AGEs and assess the biological effects of AGE removal from blood.

MATERIALS AND METHODS

sRAGE production and immobilization

sRAGE was expressed in E. Coli and purified by Ni column chromatography according to a previously published method (14). Endotoxin contamination was removed as described previously (15). After purification, sRAGE was immobilized onto agarose beads (Sepharose™ CL-4B, GE Healthcare, Inc., Piscataway, NJ) using CNBr surface activation chemistry as previously described (16). Endotoxin contamination was tested by the QCL-1000® Endpoint Chromogenic LAL Assay (Lonza Walkersville Inc., Walkersville, MD).

Characterization of bioadsorbent

The binding capacity for glycolaldehyde-derived AGE-BSA (Fitzgerald Industries International, Acton, MA) and the equilibrium dissociation constant K_D between AGE-BSA and the bioadsorbent was measured as described previously (16–18). Thermal stability of the bioadsorbent was assessed by incubating for 4 hours at 37 °C. The bioadsorbent was freeze-dried with addition of 10% sucrose, stored at 4°C, and reconstituted with water after storage for up to a year.

Small-scale, native AGE removal

The study was approved by the Institutional Review Board of Northwestern University and NorthShore University HealthSystem. Bioadsorbent, control beads, or PBS (250 μl) were incubated with human serum (diluted 1:1 with PBS) from blood donors with ESKD that had been on hemodialysis for at least one year. The supernatant was subjected to an in house-developed anti-AGE-based or sRAGE-based competitive enzyme-linked immunosorbent assay (ELISA).

Removal of AGEs by a hollow fiber bioadsorbent device

Thirty milliliters of bioadsorbent were loaded onto a customized hollow fiber device sized for use on humans (Minntech Corporation, Minneapolis, MN) that contained a bundle of polysulfone hollow fibers (0.5 μm pore size, 0.6 m^2 surface area) anchored inside a plastic cylindrical shell. After pre-equilibration, PBS (0.5 L) spiked with 4 μg/ml AGE-BSA was pumped through the device at a flow rate of 250 ml/min. The concentrations of unbound AGE-BSA in the samples were measured by the Micro bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific, Inc., Rockford, IL). Alternatively, 450 ml of human blood collected in acid-citrate-dextrose from healthy donors (Research Blood Components, LLC, Brighton, MA) was circulated through the bioadsorbent device. Blood samples were collected at multiple time points at the inlet of the device and native AGEs that remained in the plasma were measured by an anti-AGE competitive ELISA. NorthShore University

HealthSystem (Evanston, IL) performed lab tests such as CBC, chemistry and C3a and C5a complement activation. The plasma free hemoglobin level was quantified by the QuantiChrom™ hemoglobin assay kit (BioAssay Systems, Hayward, CA). A first order exponential decay mathematical model was developed to predict the AGE-BSA removal process for the typical blood volume of an adult (Supplementary Information).

Competitive anti-AGE ELISA

Anti-AGE mouse monoclonal antibody that recognizes N-carboxy-methyl lysine (CML)-AGEs (clone 6D12) (TransGenic, Inc., Kumamoto, Japan) was adsorbed to a 96-well plate pre-coated with goat anti-mouse IgG (Cayman Chemicals, Ann Arbor, MI). After blocking, plasma or serum samples and AGE-BSA standards were incubated with horseradish peroxidase-labeled AGE-BSA followed by color development with the substrate tetramethylbenzidine (TMB) (R&D systems, Inc., Minneapolis, MN). The absorbance at 450 nm was determined by a microplate reader (Tecan Group Ltd., Zurich, Switzerland). A standard curve was plotted as the percentage of absorbance range against the logarithm of standard AGE-BSA concentrations, and nonlinearly regressed by sigmoidal dose-response with variable slope.

Direct anti-AGE ELISA

Bioadsorbent or control beads previously incubated with AGE-BSA (standards) or patient serum were washed, blocked, and incubated with an anti-AGE antibody (6D12) followed by a goat anti-mouse antibody conjugated to HRP. TMB was used as the substrate, and absorbance at 450 nm was detected.

sRAGE-based competitive ELISA

Anti-human RAGE mouse monoclonal antibody (Clone 176902) (R&D systems, Inc.) was labeled with biotin using EZ-Link® Micro Sulfo-NHS-Biotinylation Kit (Thermo Fisher Scientific, Inc.). AGE-BSA was adsorbed onto a Nunc MaxiSorp® 96-well plate. The plate was washed, blocked, and incubated with serum samples or AGE-BSA standards and sRAGE. After washing, the plate was incubated with a biotinylated anti-RAGE antibody followed by HRP-conjugated streptavidin (Thermo Fisher Scientific, Inc.) and TMB substrate. The absorbance at 450 nm was determined and a standard curve was generated as described for the anti-AGE competitive ELISA.

Western blotting

Proteins were eluted from bioadsorbent or control beads by the addition of modified Laemmli's sample buffer and incubation at 95°C. Samples were separated on a 4–20% Tris-HCl gel (Bio-Rad Laboratories, Inc., Hercules, CA), transferred onto a nitrocellulose membrane (Hybond-C Extra, GE Healthcare, Inc.), and probed with an anti-AGE antibody (6D12) using the Western Breeze detection kit (Invitrogen, Inc., Carlsbad, CA).

Pro-inflammatory response of human endothelial cells

HUVECs in triplicate wells were grown to confluence in 96 well plates and treated for 24 hours with bioadsorbent, control bead, or PBS-treated serum from ESKD patients (10% total

volume in endothelial cell basal media-2) (Lonza Walkersville, Inc.) or TNFα (20 ng/ml) (positive control). Cell-bound VCAM-1 was quantified via modified ELISA (VCAM-1 Duoset kit, R&D Systems, Inc.).

Osteoclast differentiation

RAW 264.7 cells (ATCC, Rockville, MD) were cultured for 6 days with 10% fetal calf serum in the presence of various concentrations (2.5%, 5%, and 10%) of ESKD plasma or 7.5 μg/ml of AGE-BSA that were treated with the bioadsorbent or untreated. Three patients and two AGE-BSA samples were used and each sample was tested in duplicate wells. The ligand for receptor activator of NF-κB (RANKL) (25 ng/ml) was used as a positive control (Peprotech, Inc., Rocky Hill, NJ). Osteoclast formation was assessed by tartrate-resistant acid phosphatase (TRAP) staining as described (19).

Statistical analysis

Statistical significance was determined by one-way ANOVA using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA). A Bonferroni's multiple comparison test was used to compare all pairs of means, and $p < 0.05$ was considered as statistically significant. Alternatively, a student's t-test was used to compare two groups. The 95% confidence intervals of the mean are shown in Supplementary Table S5.

Results

Small scale removal of AGEs by the bioadsorbent

Glycolaldehyde-derived AGEs are one of the most reactive and toxic AGEs found *in vivo* (20). The bioadsorbent specifically bound glycolaldehyde-derived AGE-BSA (referred as AGE-BSA in this study) with a binding capacity of 0.73 ± 0.07 mg AGE-BSA / ml beads, with negligible nonspecific binding for the non-modified BSA control (Fig. 1A). Nonspecific adsorption of AGE-BSA by the control agarose beads was minimal (Fig. 1A). The equilibrium dissociation constant K_D of the bioadsorbent for AGE-BSA was 88 nM, which is consistent with reported values of AGE-BSA binding to sRAGE (14).

To determine whether treatment with the bioadsorbent would reduce endogenous serum AGE concentrations, serum from ESKD patients was incubated with PBS or saturating levels of bioadsorbent or control agarose beads. Supernatants were assessed for AGE concentration via an AGE-based competitive ELISA using an antibody that recognizes the CML epitope (Supplementary Fig. S1A), an AGE that is commonly assessed in clinical studies (10). The bioadsorbent reduced the CML-AGE concentration in the ESKD serum from 387.6 ± 57.0 μg/ml to 285.7 ± 42.97 μg/ml and there was no significant removal by the control agarose beads (Fig. 1B).

As an independent means to quantify AGE adsorption to the bioadsorbent and eliminate the potential for serum proteins to interfere with AGE quantification (i.e., competitive ELISA), an ELISA was performed directly on the bioadsorbent (Supplementary Fig. S1B, C). The direct ELISA confirmed the presence of CML-AGEs bound to the bioadsorbent with 113.5

± 11.1 μg of AGEs detected per ml of the bioadsorbent with negligible adsorption onto the control beads (Fig. 1C).

A sRAGE-based competitive ELISA (Supplementary Fig. S1D) was developed to measure the total concentration of ligands including but not limited to CML-AGEs that can bind to cell surface RAGE and trigger the activation of the pro-inflammatory NFκb pathway. The serum concentration of RAGE ligands, in which total AGEs are the majority, were two orders of magnitude higher than the CML-AGE concentration as measured by the anti-AGE competitive ELISA (Fig. 1D). The bioadsorbent depleted 57% of the total RAGE ligands from ESKD serum, whereas there was no significant removal by the control agarose beads.

Assessment of the biological effect of bioadsorbent-treated serum or plasma on cells

Endothelial cells constitutively express the RAGE receptor and respond to AGEs via activation of the NFκb pathway followed by up-regulation of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1) (9). To determine whether the bioadsorbent reduces AGE-mediated endothelial cell inflammatory activation, human umbilical vein endothelial cells (HUVECs) were treated with ESKD serum that was pre-incubated with the bioadsorbent, control beads, or PBS. Incubation with ESKD serum induced a nearly two fold increase in VCAM-1 expression that was significantly decreased when the serum was pretreated with the bioadsorbent but not the control beads (Fig. 2A).

AGE-modified proteins such as β_2-microglobulin are potent stimulators of osteoclast-mediated bone resorption and can induce the release of cytokines from cultured bone (21). To determine the impact of the bioadsorbent on bone remodeling processes, we investigated the effects of bioadsorbent-treated plasma and AGE-BSA samples on osteoclast formation. When RAW 264.7 cells were cultured with RANKL, plasma from ESKD patients, or AGE-BSA, the cells gave rise to multinucleated TRAP-positive osteoclast cells (Fig. 2B). Conversely, when the cells were incubated with ESKD plasma or AGE-BSA that had been pretreated with the bioadsorbent, osteoclast formation was abolished, similar to what was observed when culturing the cells with normal human plasma (Fig. 2B).

The presence of high molecular weight RAGE ligands has been shown to correlate with kidney dysfunction and RAGE activation in diabetic nephropathy (22). To determine the size distribution of AGEs that bound to the bioadsorbent, proteins eluted off the control and bioadsorbent beads were subjected to SDS-PAGE electrophoresis. Numerous prominent bands in the range of ~ 50–150 kDa were detected in the bioadsorbent sample, whereas very few bands were detected in the control bead sample (Fig. 2C). Western blotting with an anti-CML-AGE antibody confirmed that a subset of the AGEs eluted from the bioadsorbent but not control beads were CML-AGEs (Fig. 2C).

Efficacy of AGE-BSA removal by a hollow fiber bioadsorbent device

AGE-BSA or non-modified BSA spiked in 0.5 L of PBS were recirculated through the bioadsorbent device (Fig. 3A). While there was no significant change in BSA concentration over the time course of the experiment, 80% of the AGE-BSA was cleared from the recirculation system within 16 minutes (Fig. 3B).

A first order exponential decay mathematical model was developed to predict the AGE-BSA removal process for the typical blood volume of an adult. The exponential decay constant k_1A was constant between 0.5L and 1.0 L reservoir volumes, with coefficient of determination (r^2) values of 0.96 and 0.97, respectively. Assuming that k_1A does not change significantly during the treatment period, the model predicts 50% clearance within 1 hour, and 80% clearance within 2 hours for a 3 L reservoir volume.

Efficacy of native AGE removal in vitro and hemocompatibility of the hollow fiber bioadsorbent device

The device configuration allows AGEs to come in contact with the bioadsorbent in the shell side while keeping blood cells inside the fibers and separated from the bioadsorbent. Approximately 450 ml of human blood were recirculated through the device to assess the ability of the bioadsorbent device to remove native AGEs. By the end of 30 minutes of recirculation, the concentration of native AGEs in the system was reduced to 36% of initial (Fig. 4A). The bioadsorbent cleared 7.4 ± 2.8 mg of AGEs from the blood with no further AGE removal observed after 30 minutes of recirculation (n = 4 independent experiments). In contrast, there was no significant nonspecific absorption of AGEs by the device when loaded with control beads (Fig. 4A). Furthermore, a direct ELISA performed on the bioadsorbent and control beads isolated from the device showed an approximately three-fold increase in AGE binding to the bioadsorbent compared to the control beads (Fig. 4B).

In regards to the hemocompatibility of the bioadsorbent device, there was no significant change in blood chemistry such as electrolytes, total protein, and albumin concentration. The red blood cell (RBC) and white blood cell (WBC) counts dropped less than 4% and 11%, respectively, after two hours of recirculation (Table 1). The platelet count decreased to 73% of initial, but the final count of 216 thousands/μL was within the normal range (150 – 400 thousands/μL) (Table 1). Although there was a slight increase in the plasma free hemoglobin (PFH) level due to hemolysis (Table 1), the accumulated hemolysis rate was 0.11 mg PFH / dL plasma per pass, well below the safety criteria (2.6 mg PFH / dL per pass) (23). After 10 minutes of recirculation, the plasma level of complement cascade component C3a significantly increased (Table 1).

The biocompatibility of the bioabsorbent or a control device was also evaluated in a porcine extracorporeal circulation model. During a 90 minute extracorporeal recirculation, there were no significant changes in most of the CBC and blood chemistry measurements such as RBC, hemoglobin, total protein, albumin, liver and renal panels (Supplementary Tables S1–4). Although the platelet counts, WBC, Gamma-glutamyl Transferase (GGT) and glucose were affected in some experiments with the bioadsorbent device, the levels remained well within the normal range for the minipig or consistent with the data obtained from the Cuprophan control device (Supplementary Tables S1–4). In summary, there were no severe adverse responses to the bioadsorbent device.

Assessment of thermal stability and storage condition of the bioadsorbent

In order for the bioadsorbent device to be clinically relevant, it should withstand the typical operating and storage conditions expected in a hemodialysis session as well as long-term

storage. The binding capacity was maintained after incubation of the bioadsorbent for 4 hours at 37°C (0.66 ± 0.02 and 0.60 ± 0.01 mg AGE-BSA / ml beads, before and after, respectively) (Fig. 5A). Furthermore, the bioadsorbent maintained its original spherical morphology (Fig. 5B) and showed no significant changes in the diameters of the bioadsorbent beads before and after freeze-drying (62.9 ± 2.2 μm and 62.0 ± 1.8 μm, respectively). After one year of storage at 4°C, the binding capacity of the bioadsorbent was fully recovered after rehydration (0.69 ± 0.03 and 0.73 ± 0.01 mg AGE-BSA / ml beads, before and after, respectively) (Fig. 5C).

DISCUSSION

A polysulfone hollow fiber device containing agarose-immobilized RAGE was developed as a potential therapeutic strategy to selectively remove AGEs from human blood and thereby mitigate the activation of RAGE-mediated pro-inflammatory pathways. Data from several independent assays that detected CML-AGEs (i.e., competitive and direct AGE-based ELISAs; Western blotting of proteins eluted off the bioadsorbent) as well as total AGEs (i.e., competitive sRAGE-based ELISA) provide compelling evidence that the bioadsorbent was capable of removing endogenous AGEs from the serum of ESKD patients. The bioadsorbent was shown to bind high molecular weight ligands from ESKD serum (~ 50–150 kDa). Removal of such proteins by the bioadsorbent inhibited the inflammatory activation of endothelial cells and osteoclast differentiation, suggesting that the absorbed proteins were physiologically relevant. Our data are consistent with a recent report in which high molecular weight RAGE ligands (> 30 kDa) shown to be highly prominent in the serum of patients with diabetic nephropathy were responsible for RAGE-induced activation of mesangioblasts (22).

When human blood was recirculated through the device loaded with bioadsorbent, endogenous AGEs were reduced by ~ 60%, confirming that efficient AGE removal was achieved on a larger scale. AGE removal reached a plateau at 30 minutes, suggesting that AGE binding may have saturated the bioadsorbent. Alternatively, the plateau may reflect the inherent binding affinity of CML-AGE for RAGE (24). The device may be packed with more bioadsorbent beads to further deplete AGE levels and potentially scale up for human use. The thermal and long-term stability of the bioadsorbent device offer additional advantages for clinical translation. In studies of hemocompatibility, human whole blood recirculated through the device showed normal blood chemistry tests and blood cell counts by the completion of the experiment. Furthermore, total protein and albumin concentrations were not significantly affected by the procedure, which again confirmed that nonspecific protein absorption by the bioadsorbent is negligible. Activation of the complement cascade is typical for extracorporeal circulation involving devices that contain hydroxyl groups, including regenerated cellulose dialyzers (25). The plasma level of complement cascade component C3a significantly increased in our experiments, suggesting that the complement system had been activated, possibly by the hydroxyl groups on agarose beads. However, there is no substantial evidence linking complement activation to a detrimental clinical outcome in patients (25). If necessary, the agarose beads can be modified with maleic anhydride, which has been reported to greatly reduce complement activation without losing the immobilization capability via CNBR chemistry (26). Also other porous particles made

from materials such as polyacrylamide and acetylated cellulose that are non-complement activating can be employed to immobilize RAGE. Importantly, the endotoxin level in the agarose-immobilized RAGE (bioadsorbent) met the FDA's requirement of medical devices (less than 0.5 EU/ml endotoxin in the eluate) for use on humans.

Due to the lack of a validated and robust large animal model for AGE-mediated chronic inflammation and the lack of available reagents to quantify AGE removal in pig, we were not able to assess the biological efficacy of AGE removal *in vivo*. However, hematologic and plasma biochemistry data obtained from preliminary extracorporeal procedures in the Yucatan minipigs showed that the biocompatibility of the bioadsorbent device was comparable to a clinical grade Cuprophan dialyzer. No significant changes in most of the CBC and blood chemistry tests were detected. However, A transient drop in WBC at 10 minute after the start of extracorporeal procedures was observed in both the bioadsorbent device and the Cuprophan control, a phenomenon usually correlated with activation of the complement cascade (25). The proposed modifications of the bioadsorbent described above may increase the biocompatibility of the device. Clearly, further studies in large animal models need to be performed to evaluate the safety of a bioadsorbent device that effectively removes AGEs.

The key components of the bioadsorbent device, porous beads and hollow fiber membranes, have been widely used in clinics for blood purification. The bioadsorbent device could be easily integrated into a standard dialysis circuit, similar to procedures involving charcoal hemoperfusion during dialysis. Therefore, compared to pharmaceutical approaches to normalize AGEs level including systemic injection of sRAGE, the method herein described is a new and completely different therapeutic strategy that anticipates less negative systemic side effects.

AGEs are a heterogenous class of compounds including non-fluorescent non-crosslinked species such as CML and pyrraline, and fluorescent crosslinked species such as pentosidine and crossline. The CML-protein adduct is the main epitope of commercially available antibodies used for the quantification of AGEs (27), including the anti-AGE antibody used in the competitive and direct ELISA in this study. Although it has been well documented that AGEs are deleterious to human health (1, 8), the species responsible for predicting or promoting cardiovascular events in patients with CKD remain controversial (1). Furthermore, the full complement of toxic AGEs involved in kidney damage is not known (28). The immobilized RAGE bioadsorbent can potentially be used as a tool to remove AGEs from serum, and with further separation and sequencing, identify the type of species that are relevant to diseases such as kidney failure, diabetes, cardiovascular disease, or Alzheimer's disease (29, 30).

CONCLUSION

The data reported in this proof-of-principle study confirm the feasibility of a RAGE-receptor based bioadsorbent device to selectively remove AGEs from human blood. The use of a RAGE-based bioadsorbent may ultimately lead to the identification of novel mechanisms by

which AGEs exert their effects and new strategies (extracorporeal, pharmacological, or genetic) to inhibit AGE-induced systemic inflammatory activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Busch M, Franke S, Rüster C, Wolf G. Advanced glycation end-products and the kidney. Eur J Clin Invest. 2010; 40:742–755. [PubMed: 20649640]
2. Kilhovd BK, Juutilainen A, Lehto S, Ronnemaa T, Torjesen PA, Hanssen KF, et al. Increased serum levels of advanced glycation endproducts predict total, cardiovascular and coronary mortality in women with type 2 diabetes: a population-based 18 year follow-up study. Diabetologia. 2007; 50:1409–1417. [PubMed: 17479244]
3. Nin JW, Jorsal A, Ferreira I, Schalkwijk CG, Prins MH, Parving HH, et al. Higher plasma levels of advanced glycation end products are associated with incident cardiovascular disease and all-cause mortality in type 1 diabetes: a 12-year follow-up study. Diabetes Care. 2011; 34:442–447. [PubMed: 21270199]
4. United States Renal Data System. National Institutes of Health. Bethesda, MD: National Institute of Diabetes and Digestive and Kidney Diseases; 2009. Annual Data Report: Atlas of Chronic Kidney Disease and End-Stage Renal Disease.
5. Meerwaldt R, Zeebregts CJ, Navis G, Hillebrands JL, Lefrandt JD, Smit AJ. Accumulation of advanced glycation end products and chronic complications in ESRD treated by dialysis. Am J Kidney Dis. 2009; 53:138–150. [PubMed: 19036487]
6. Schmidt AM, Vianna M, Gerlach M, Brett J, Ryan J, Kao J, et al. Isolation and characterization of two binding proteins for advanced glycosylation end products from bovine lung which are present on the endothelial cell surface. J Biol Chem. 1992; 267:14987–14997. [PubMed: 1321822]
7. Kislinger T, Fu C, Huber B, Qu W, Taguchi A, Du Yan S, et al. N(epsilon)-(carboxymethyl)lysine adducts of proteins are ligands for receptor for advanced glycation end products that activate cell signaling pathways and modulate gene expression. J Biol Chem. 1999; 274:31740–31749. [PubMed: 10531386]
8. Yan S, Ramasamy R, Schmidt A. Receptor for AGE (RAGE) and its ligands—cast into leading roles in diabetes and the inflammatory response. J Mol Med. 2009; 87:235–247. [PubMed: 19189073]
9. Bierhaus A, Nawroth P. Multiple levels of regulation determine the role of the receptor for AGE (RAGE) as common soil in inflammation, immune responses and diabetes mellitus and its complications. Diabetologia. 2009; 52:2251–2263. [PubMed: 19636529]
10. Engelen L, Stehouwer CD, Schalkwijk CG. Current therapeutic interventions in the glycation pathway: evidence from clinical studies. Diabetes Obes Metab. 2012
11. Wendt T, Harja E, Bucciarelli L, Qu W, Lu Y, Rong LL, et al. RAGE modulates vascular inflammation and atherosclerosis in a murine model of type 2 diabetes. Atherosclerosis. 2006; 185:70–77. [PubMed: 16076470]
12. Ekong U, Zeng S, Dun H, Feirt N, Guo J, Ippagunta N, et al. Blockade of the receptor for advanced glycation end products attenuates acetaminophen-induced hepatotoxicity in mice. J Gastroenterol Hepatol. 2006; 21:682–688. [PubMed: 16677153]
13. Mitsuhashi T, Li YM, Fishbane S, Vlassara H. Depletion of reactive advanced glycation endproducts from diabetic uremic sera using a lysozyme-linked matrix. J Clin Invest. 1997; 100:847–854. [PubMed: 9259584]

14. Wilton R, Yousef MA, Saxena P, Szpunar M, Stevens FJ. Expression and purification of recombinant human receptor for advanced glycation endproducts in Escherichia coli. Protein Expr Purif. 2006; 47:25–35. [PubMed: 16510295]
15. Reichelt P, Schwarz C, Donzeau M. Single step protocol to purify recombinant proteins with low endotoxin contents. Protein Expr Purif. 2006; 46:483–488. [PubMed: 16290005]
16. Ameer GA, Grovender EA, Ploegh H, Ting D, Owen WF, Rupnick M, et al. A novel immunoadsorption device for removing [bgr]2-microglobulin from whole blood. Kidney Int. 2001; 59:1544–1550. [PubMed: 11260419]
17. Daniels CM, Woolverton EM, Sprague SM, Ameer GA. Assessment of the Stability of an Immunoadsorbent for the Extracorporeal Removal of Beta-2-Microglobulin from Blood. Blood Purif. 2005; 23:287–297. [PubMed: 15942167]
18. Grovender EA, Cooney CL, Langer R. Immunoadsorption Model for a Novel Fluidized-Bed Blood Detoxification Device. AIChE. 2002; 48:2357–2365.
19. Menaa C, Reddy SV, Kurihara N, Maeda H, Anderson D, Cundy T, et al. Enhanced RANK ligand expression and responsivity of bone marrow cells in Paget's disease of bone. J Clin Invest. 2000; 105:1833–1838. [PubMed: 10862799]
20. Takeuchi M, Yamagishi S. TAGE (toxic AGEs) hypothesis in various chronic diseases. Medical Hypotheses. 2004; 63:449–452. [PubMed: 15288366]
21. Miyata T, Kawai R, Taketomi S, Sprague SM. Possible involvement of advanced glycation end-products in bone resorption. Nephrol Dial Transplant. 1996; 11(Suppl 5):54–57. [PubMed: 9044308]
22. Penfold SA, Coughlan MT, Patel SK, Srivastava PM, Sourris KC, Steer D, et al. Circulating high-molecular-weight RAGE ligands activate pathways implicated in the development of diabetic nephropathy. Kidney Int. 2010; 78:287–295. [PubMed: 20463655]
23. Ameer GA, Harmon W, Sasisekharan R, Langer R. Investigation of a whole blood fluidized bed Taylor-Couette flow device for enzymatic heparin neutralization. Biotechnol Bioeng. 1999; 62:602–608. [PubMed: 10099569]
24. Valencia JV, Weldon SC, Quinn D, Kiers GH, DeGroot J, TeKoppele JM, et al. Advanced glycation end product ligands for the receptor for advanced glycation end products: biochemical characterization and formation kinetics. Anal Biochem. 2004; 324:68–78. [PubMed: 14654047]
25. Holmes CJ. Hemodialyzer Performance: Biological Indices. Artif Organs. 1995; 19:1126–1135. [PubMed: 8579525]
26. Fowers KD, Janatova J. Specific inhibition of C3 to facilitate general complement inhibition on endotoxin affinity sorbents for apheresis applications. Biomaterials. 2001; 22:1749–1761. [PubMed: 11396878]
27. Ikeda K, Higashi T, Sano H, Jinnouchi Y, Yoshida M, Araki T, et al. Nε-(Carboxymethyl)lysine Protein Adduct Is a Major Immunological Epitope in Proteins Modified with Advanced Glycation End Products of the Maillard Reaction†. Biochemistry. 1996; 35:8075–8083. [PubMed: 8672512]
28. Williams ME. New therapies for advanced glycation end product nephrotoxicity: current challenges. Am J Kidney Dis. 2003; 41:S42–S47. [PubMed: 12612951]
29. Semba RD, Fink JC, Sun K, Windham BG, Ferrucci L. Serum carboxymethyl-lysine, a dominant advanced glycation end product, is associated with chronic kidney disease: the Baltimore longitudinal study of aging. J Ren Nutr. 2010; 20:74–81. [PubMed: 19853477]
30. Takeuchi M, Yamagishi S. Possible involvement of advanced glycation end-products (AGEs) in the pathogenesis of Alzheimer's disease. Curr Pharm Des. 2008; 14:973–978. [PubMed: 18473848]

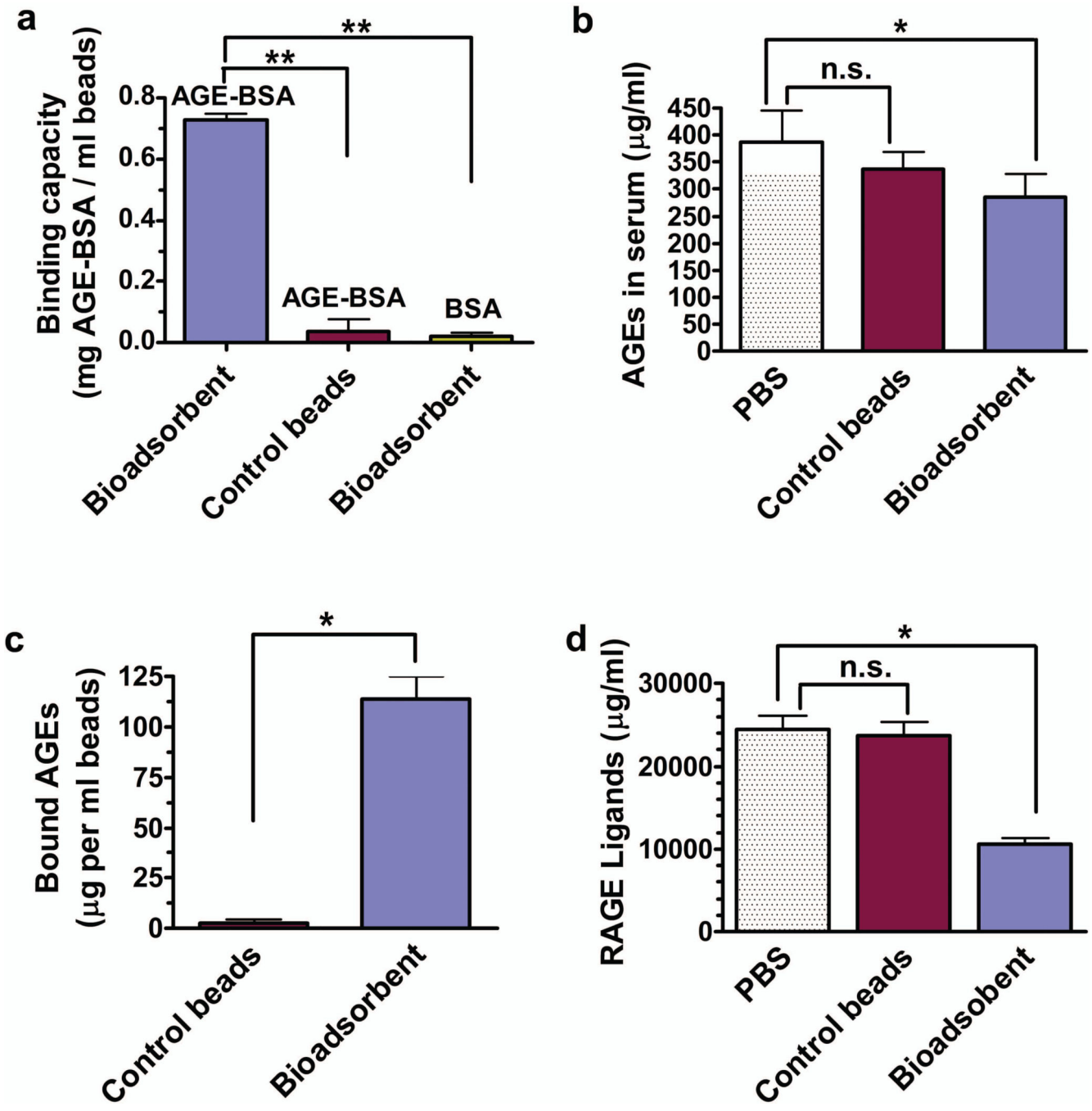


FIG. 1.

The bioadsorbent selectively removes exogenous AGE-BSA from saline and removes endogenous AGEs or RAGE ligands from human serum in a batch mode. (A) The bioadsorbent preferentially bound AGE-BSA (blue bar) with negligible nonspecific binding for non-modified BSA (yellow bar). (B) The bioadsorbent (blue bar), but not the control agarose beads (red bar) induced a significant reduction in native CML-AGE concentration in ESKD serum, as assessed by anti-AGE-based competitive ELISA. n = 9 ESKD blood donors. (C) Endogenous CML-AGEs from pooled ESKD serum showed significant specific

adsorption onto the bioadsorbent (blue bar). Binding of AGEs to controls beads was minimal (red bar). n = 9 blood donors. (D) The bioadsorbent (blue bar) but not the control agarose beads (red bar), depleted total RAGE ligands from ESKD serum, as assessed by sRAGE-based competitive ELISA. n = 8 blood donors. *$p < 0.05$; **$p < 0.001$; n.s. = non-significant ($p > 0.05$). Error bars represent standard error of the mean (SEM).

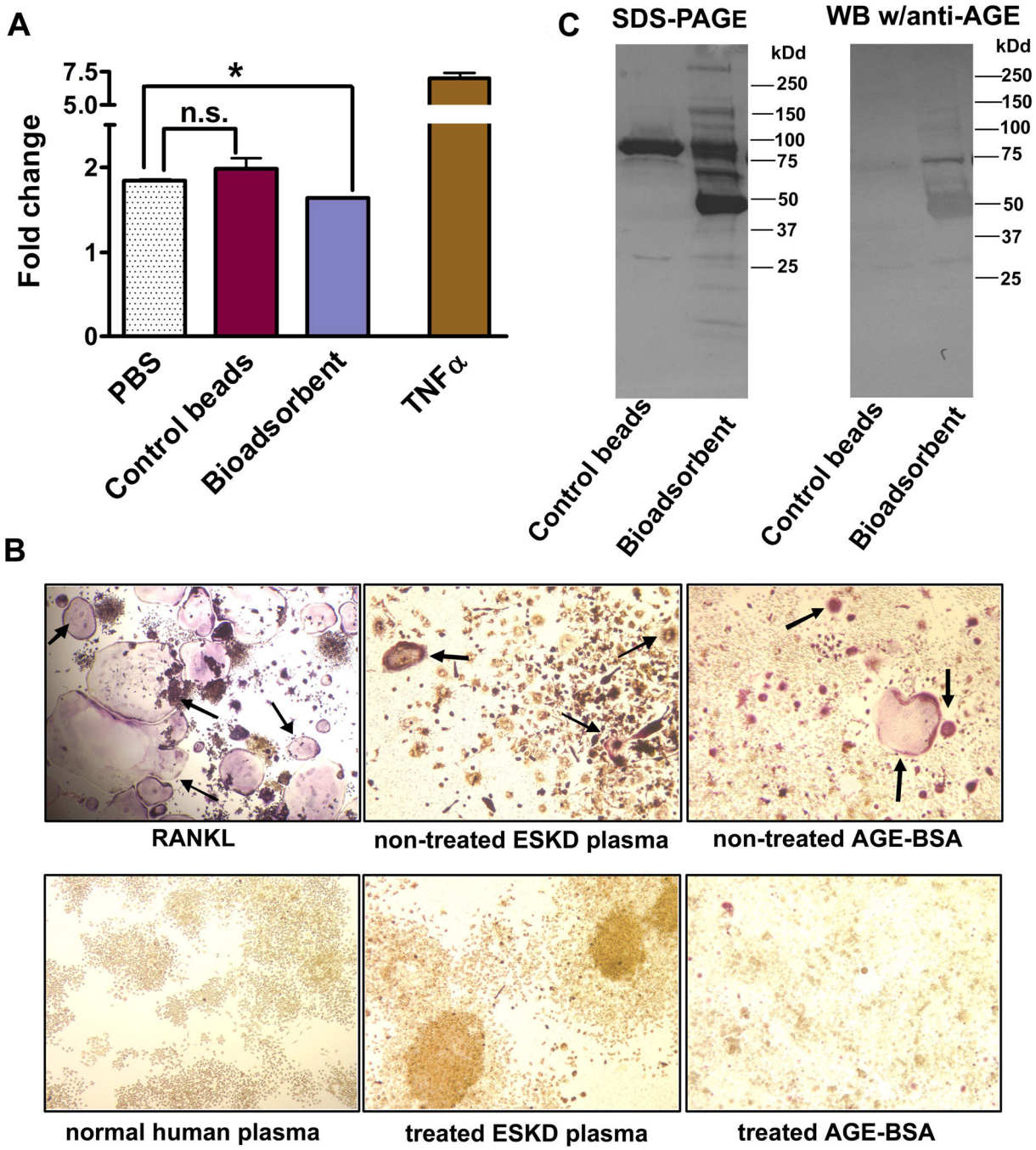


FIG. 2.

The bioadsorbent reduces AGE-induced inflammation and inhibits osteoclast differentiation *in vitro*. (A) Bioadsorbent-treated serum significantly reduced expression of the inflammatory marker VCAM-1 in human endothelial cells. Cells were stimulated with TNFa (tan bar) as a positive control. White, red and blue bars represent ESKD serum treated with PBS, control agarose beads and the bioadsorbent, respectively. Data are represented as mean ± SEM of 2 independent experiments that were performed in triplicate. *$p<0.05$, n.s. = non-significant ($p > 0.05$). (B) Treatment of AGE-BSA or plasma samples from ESKD patients

with the bioadsorbent inhibited osteoclast differentiation. RAW 264.7 cells were incubated with bioadsorbent-treated or non-treated AGE-BSA, normal plasma, or ESKD plasma. The osteoclast stimulator RANKL was included as a positive control. The arrows represent osteoclasts, identified as multinucleated TRAP-positive cells. (C) Proteins eluted off of bioadsorbent or control beads were separated by SDS-PAGE electrophoresis and stained with Coomassie Blue (*Left*) or probed with an anti-AGE antibody in a Western blot (*Right*).

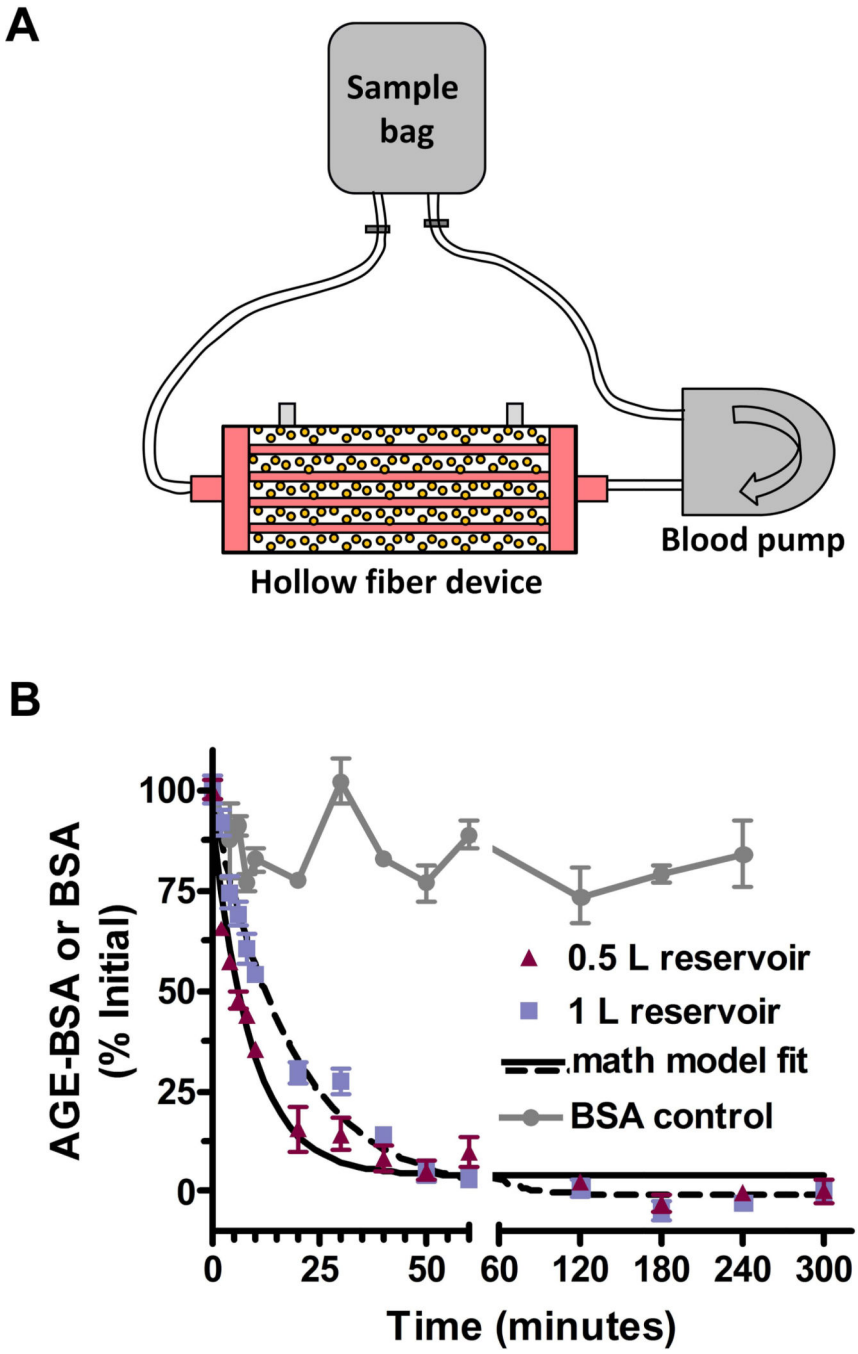


FIG. 3.

A hollow fiber device containing the bioadsorbent is able to specifically remove spiked AGE-BSA from saline recirculated at a flow rate of 250 ml/min. (A) Schematic of the experimental setup. (B) Red triangles and blue squares represent experimental AGE-BSA removal from 0.5 L and 1 L reservoirs, respectively. Gray circles represent the removal of unmodified BSA. The data were fitted by a first order exponential decay mathematical model. 50% clearance was achieved within 6 minutes for the 0.5 L reservoir and 12 minutes for the 1 L reservoir. Error bars represent SEM. n = 2.

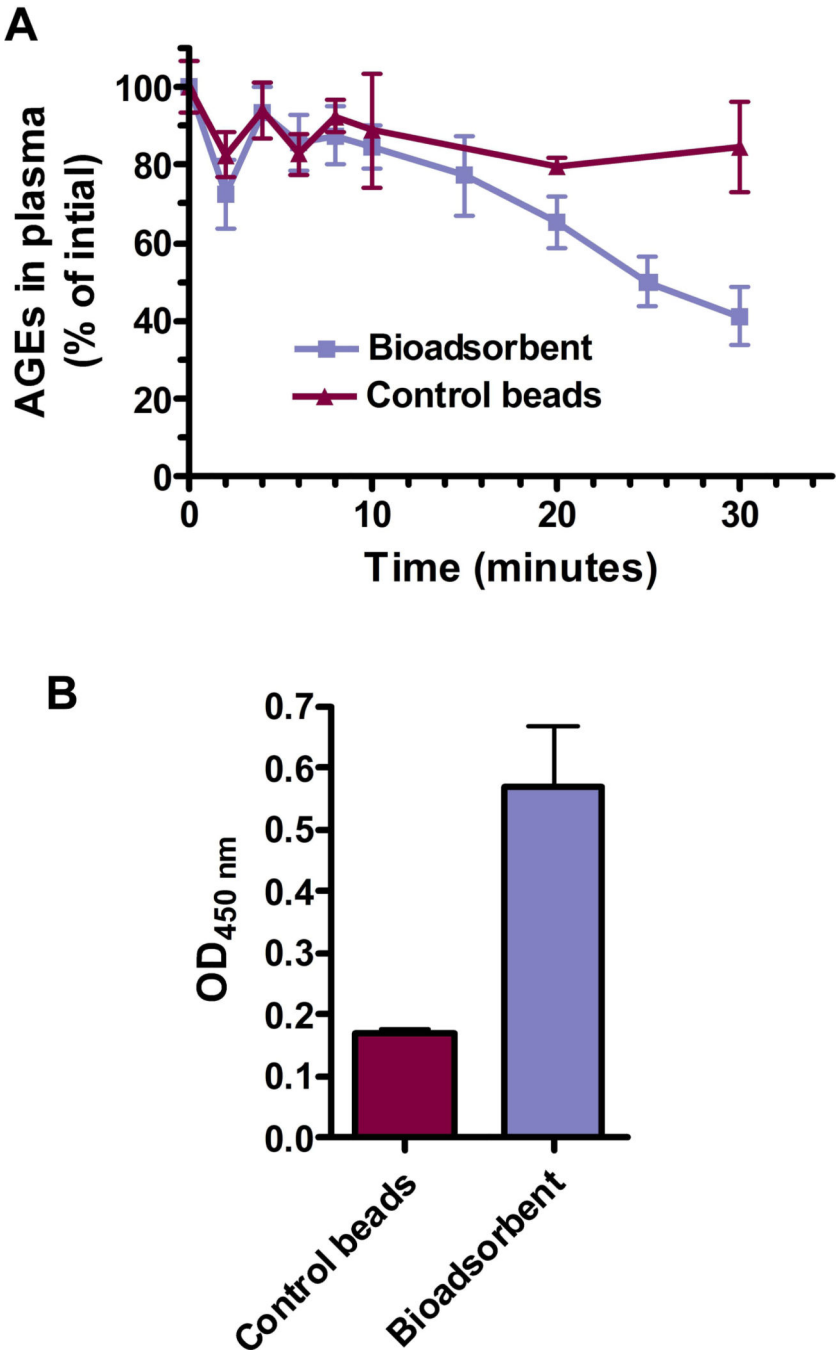
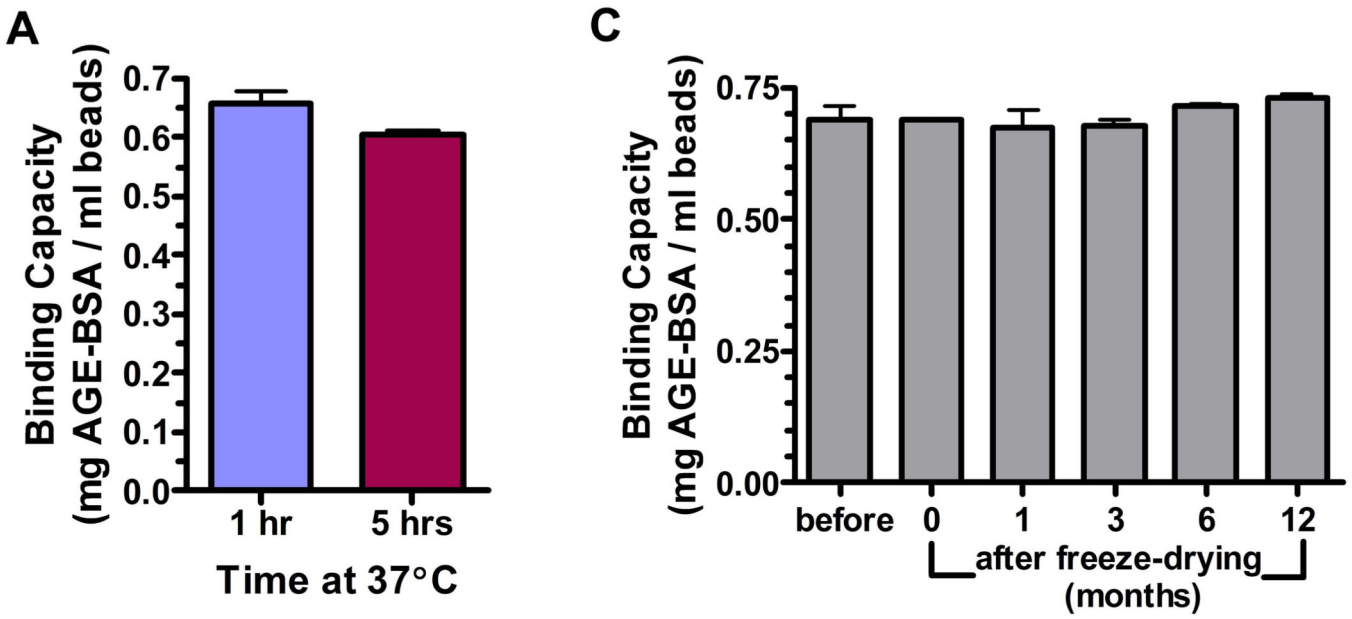


FIG. 4.

The bioadsorbent loaded in a hollow fiber device specifically removes endogenous AGEs from *in vitro* recirculation of human blood at 250 ml/min. (A) AGEs remaining in blood as assessed via anti-AGE-based competitive ELISA. Blue and red symbols represent AGEs removal by the bioadsorbent (n = 12) or control agarose beads (n = 3) loaded in the device, respectively. Error bars represent SEM. (B) Adsorbed AGEs on the beads after 30 minutes of recirculation were measured by direct ELISA. Data show mean ± SEM of one representative of 3 independent experiments.



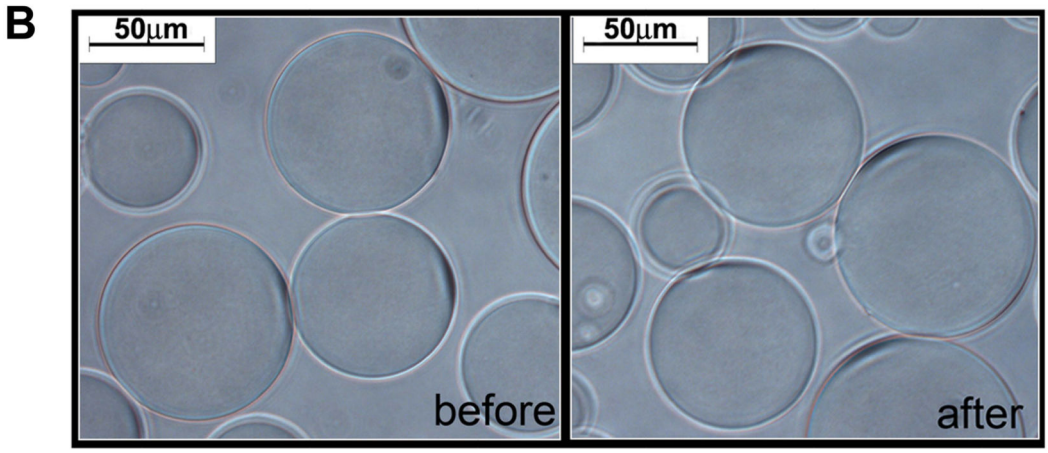


FIG. 5.

The bioadsorbent is thermally stable and can be freeze-dried for long term storage. (A) The binding capacity was maintained after exposure to 37 °C for 5 hours, $p > 0.05$. (B) Microscope images of bioadsorbent before and after freeze-drying. Freeze-drying did not change bead morphology. Scale bar represents 50 mm. (C) The bioadsorbent did not lose binding capacity after freeze-drying and rehydration, $p > 0.05$ across all groups. Bioadsorbent was reconstituted with water after being stored at 4 °C for up to a year. Error bars represent SEM.

Table 1

Hemocompatibility of human whole blood with the bioadsorbent device

Time (minutes)	WBC (% initial)	RBC (% initial)	Platelet (% initial)	PFH (mg/dL)	C3a (ng/ml)	C5a (ng/ml)
0	100	100	100	15	675	8.4
2	95	97	88	18		
10	97	96	86	16	>1100	9.8
120	89	97	73	23		
240	89	97	72	28	672	14.5