Research Article

Ratiometric fluorescent L-arginine and L-asparagine biosensors based on the oxazine 170 perchlorate-ethyl cellulose membrane

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Abbreviations: AFM, atomic force microscopy; **ANOVA**, one-way analysis of variance; **Arg**, arginine; **Asn**, asparagine; **EC**, ethyl cellulose; **LOD**, limit of detection; **O17**, oxazine 170 perchlorate; **PU**, polyurethane

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Practical application

The present work describes a simple construction and application of a ratiometric fluorescent biosensor for the determination of L-Arg and L-Asn in biological materials. This ratiometric fluorescence technique can provide a built-in self-correction to the environmental effects, offering advantages in terms of sensitivity, accuracy and reversibility. Ratiometric fluorescent biosensors with immobilized enzymes and an ammonia-sensing membrane will open up new opportunity for analytical sensing and optical imaging in clinical chemistry and biological processes.

Abstract

Ratiometric fluorescent L-arginine (Arg) and L-asparagine (Asn) biosensors were developed using an oxazine 170 perchlorate (O17)-ethyl cellulose (EC) membrane and the enzymes entrapped into the matrix of ethyl cellulose and hydrogel polyurethane. The sensing principles were based on the hydrolysis reactions of urea and L-Arg under the catalysis of the urease and arginase to produce ammonia in the case of an L-Arg-sensing membrane and also on the hydrolysis reaction of L-Asn under the catalysis of asparaginase in the case of an L-Asn-sensing membrane. The O17-EC membrane reacted with the ammonia produced from the hydrolysis reactions and changed the fluorescence intensities at λ_{em}=565 and 625 nm. The ratio of the fluorescence intensities at λ_{em}=565 and 625 nm was proportional to the concentrations of L-Arg or L-Asn in the range of 0.1-10 mM. The limit of detection of the L-Arg- and L-Asn-sensing membranes was 0.082±0.0014 and 0.074±0.0023 mM, respectively. The sensing membranes also showed good quality in terms of response time, reversibility, and stability. The interference study demonstrated that some components such as amino acids had little negative effects on the performance of the sensing membranes for the detection of L-

Arg and L-Asn. These simple and sensitive ratiometric fluorescent sensing membranes provide a basic or comprehensive method for detecting L-Arg and L-Asn in blood and urine samples as well as in the fermentation processes.

1 Introduction

L-Arginine (Arg) is the most basic natural amino acid and has the highest proton affinity among amino acids [1]. It serves as a precursor of the biosynthesis of polyamines and nitric oxide. It also plays an important role in cell division, the healing of wounds, the removal of ammonia from the body, the releasing of hormones and other fields of medical science [2]. Lack of L-Arg can lead to heart problem, hormonal and sexual disorders and many other diseases [3]. L-Arg concentrations are in the range of 0.06-0.4 mM in healthy body plasma samples. Average dose of 30 mM L-Arg is taken daily as diet and some proportion of L-Arg can be found in urine sample. Therefore, the levels of L-Arg in blood serum and urine sample are of great importance and the key indicator in healthcare diagnostics and treatment. High amounts of L-Arg (up to 330 mM) could be also obtained through the fermentation processes of *Corynebacterium* spp. [4,5], while they are found in plants, particularly in roots and reserve.

L-Asparagine (Asn) is the main amino acid that forms acrylamide by the Millard reaction in backed chemistry [6]. It is not essential for humans, indicating that it can be synthesized from central metabolic pathway intermediates; however, it is required for the development of brain and regulates the equilibrium of central nervous system [7]. It is also hydrolyzed to L-aspartate, which undergoes transamination to form glutaminate and oxaolacetate from α-ketoglutarate. It is considered as an effective drug to lower blood pressure, anti-peptic ulcer and relief gastric dysfunction [8]. Therefore, it is quite meaningful to detect the concentration of L-Asn at different stages of physiological changing procedure. L-Asn concentration in a healthy adult is in the range of 0.03-0.06 mM., but they

may rise to above 0.1-0.4 mM in certain pathological conditions. L-Asn is also one of the most suitable nitrogen sources for the production of bioactive metabolites and proteins by *Streptomyces* sp. 201 [9] and *Dictyostelium discoideum* [10].

For the optimal growth of microorganisms and the enhancement of the production it is necessary to monitor the concentrations of L-Arg and L-Asn in fermentation processes with sensors or other on-line monitoring systems. A large number of methods have been developed to analyze the concentrations of L-Arg and L-Asn in biological samples, including ion exchange chromatography [11], spectrophotometry [12], capillary electrophoresis [13], and gas chromatography [14]. Moreover, the determination of L-Arg and L-Asn based on high-performance liquid chromatography should be followed by fluorescence detection using post-column derivatization with ninhydrin [15]. This chromatographic method is time-consuming, expensive, and requires skillful labor techniques. To overcome the disadvantage of the chromatographic method, a few biosensors for the detection of L-Arg or L-Asn have been developed by using either a bienzymatic system with arginase and urease [16] or one enzyme such as L-arginine oxidase [17] and asparaginase [18]. The enzymes were also immobilized in some supporting materials and combined with a flow injection analysis system to determine the concentrations of L-Arg or L-Asn continuously [19,20]. Some transducers such as potentiometric or conductometric electrode and amperometric transducer have been also used to get analytical signals from the enzymatic reactions for the detection of L-Arg and L-Asn. For the quantitative determination of L-Arg, a highly sensitive conductometric biosensor has been developed by co-immobilizing arginase and urease into a single bioselective membrane on the working sensor [1]. A novel amperometric L-Arg biosensor based on recombinant human arginase I isolated from the recombinant yeast *Hansenula polymorpha* and commercial urea was explored by placing the enzymes onto a polyaniline-nafion composite platinum electrode and then covering with a calcium alginate gel [21]. A urease-based ion-selective field effect transistor biosensor for L-Arg detection has been also proposed based on the urease inhibition effects by L-Arg [22]. For the detection of L-Asn, a

miniaturized fiber optic biosensor was developed by immobilizing L-asparaginase and absorptive pH sensitive indicator onto plastic chips of 5 mm [18]. An ammonium-selective electrode was used to develop a biosensor for L-Asn by immobilizing asparaginase in the front of the electrode [23]. Recombinant asparaginase from a strain of *Erwinia carotovora* was used to assemble a microplate-based biosensor by immobilizing the enzyme into the well of a microplate in a 96-well format and based on the colorimetric measurement of ammonia formation using Nessler's reagent [25].

In this study, we used a ratiometric fluorescent biosensor for the determination of L-Arg and L-Asn. The major advantage of this ratiometric fluorescent sensor is its good stability. The ratiometric fluorescent biosensors for the detection of L-Arg and L-Asn were fabricated based on the O17-EC membrane from our previous research [26] for a ratiometric ammonia-sensing membrane.

The detection of L-Arg was based on the coupled reaction with arginase and urease, in which arginase catalyses the hydrolysis of L-Arg and the produced urea produces ammonium ion under urease-catalyzed hydrolysis:

$$\text{L-Arginine} + H_2O \xrightarrow{\text{Arginase}} \text{L-ornithine} + \text{urea}$$

$$\text{Urea} + 2\,H_2O + H^+ \xrightarrow{\text{Urease}} 2NH_4^+ + HCO_3$$

The sensing membrane for L-Arg is a double layer consisting of the O17-EC membrane as an optical transducer and a layer of the enzymes (i.e., urease and arginase) entrapped in an ethyl cellulose matrix.

The detection of L-Asn is based on the hydrolysis of L-Asn by asparaginase to produce ammonia as one of the primary products:

$$\text{L-Asparagine} + H_2O \xrightarrow{\text{Asparaginase}} \text{L-aspartate} + NH_4^+$$

The sensing membrane for L-Asn is a double layer consisting of the O17-EC membrane as an optical transducer and a layer of the enzyme (i.e., asparaginase) entrapped in a matrix of ethyl cellulose (EC) and poly hydrogel (PU). Ammonium ions are detected by the O17-EC membrane and leads to change the fluorescence intensities at λ_{em}=565 and 625 nm. The data collected from the ratio of the fluorescence intensities at λ_{em}=565 nm (FI_{565}) and λ_{em}=625 nm (FI_{625}) are proportional to the concentrations of L-Arg and L-Asn. In this study, the properties of the L-Arg- and L-Asn-sensing membrane were investigated, and their response to L-Arg and L-Asn was also investigated by ratiometric calculation.

2 Materials and Methods

2.1 Materials/reagents

Oxazine 170 perchlorate (O17), ethyl cellulose (EC), urease (59,400 unit(U)/g-solid, from *Canavalia ensijormis*(Jack bean)), asparaginase (100-300 U/mg-protein, *Escherichia coli*), L-Arg, L-Asn and some L-amino acids such as L-lysine, and L-methionine were purchased from Sigma-Aldrich Chemical Co (Seoul, Korea). Arginase (105 U/mg-solid, from Bovine Liver) was purchased from Calzyme Lab.

Inc. (San Louis Obispo, CA., USA), and hydrogel polyureathane (PU) was purchased from Advance Source Biomaterials Co. (USA). Saccharides such as glucose and arabinose were also purchased from Sigma-Aldrich Chemical Co (Seoul, Korea). Other analytical-grade chemicals such as ammonium sulfate, sodium phosphate, sodium chloride, sodium hydroxide, hydrochloric acid, and urea were used without further purification.

2.2 Preparation of L-Arg- and L-Asn-sensing membranes

The O17-EC membranes were first prepared as reported in our previous study [26] by mixing 15 μL of O17 stock (2 mg mL^{-1}) with 300 μL of EC (10.0% w/v) in ethanol. The mixture was incubated for 4 h at room temperature, and then 10 μL of the mixture were coated on the bottom of one well in the 96-well microtiter plate (NUNC Co., Copenhagen, Denmark). Afterwards, the O17-EC membrane was dried at 60 ^{o}C for 12 h. For the preparation of the L-Arg-sensing membrane 20 μL of EC (10% w/v) was covered over the O17-EC membrane in a well. Then, 15 μL of an enzyme mixture containing urease (20 U) and arginase dissolved in 10 mM phosphate buffer saline (PBS) was placed into the well with EC (10% w/v). The enzymes were then entrapped in the ethyl cellulose (EC) matrix and immobilized over the O17-EC membrane. The L-Arg-sensing membrane containing two enzymes was incubated for 72 h at 4 ^{o}C.

The L-Asn-sensing membrane was prepared with 20 μL of EC (10% w/v) and PU solution covered over the O17-EC membrane in a well, similar to the preparation of the L-Arg-sensing membrane. Only one enzyme, asparaginase dissolved in 10 mM PBS (pH 7.4) was placed into the well with EC (10% w/v) and PU. The enzyme was then entrapped into the EC and PU matrix and immobilized over the O17-EC membrane. The L-Asn-sensing membrane containing asparaginase was

incubated for 72 h at 4 °C. The surface morphologies of the L-Arg- and L-Asn-sensing membranes were identified by atomic force microscopy (AFM).

2.3 Measurements of L-Arg and L-Asn

The concentrations of L-Arg and L-Asn were measured in the range of 0.1-10 mM. Data was collected from the fluorescence intensity of the L-Arg-and L-Asn-sensing membranes at the two emission wavelengths (λ_{em}=565 and 625 nm) at an excitation wavelength of 460 nm (λ_{ex}=460 nm). The fluorescence spectra for detecting L-Arg and L-Asn were measured using a multifunctional fluorescence microtiter plate reader (Saphire^2, Tecan GmbH, Wien, Austria). Limit of detection is calculated based on the ratio of standard deviation (SD) of the blank sample to slope value (Sl) of a linear calibration curve, i.e. LOD=3.3* SD/Sl

The L-Arg-sensing membranes immobilized with various amounts of arginase and 20 U of urease were used for the measurements of different L-Arg concentrations. The arginase amount for immobilization was optimized with 10 U, 20 U, 30 U, 35 U and 40 U of arginase and with a fixed amount (20 U) of urease. The immobilization efficiency of two enzymes (urease and arginase) into the L-Arg-sensing membrane was calculated by dividing the amount of immobilized enzymes by the total amount of the enzymes used for immobilization, as shown in our previous study [27]. The apparent kinetic parameters, apparent maximal reaction rate (V_{max}^{app}), and Michaelis-Menten constant (K_m^{app}) of the immobilized arginase and urease were determined from the Hanes plot based on the ratio of the fluorescence intensities at λ_{em}=565 and 625 nm.

The optimal amount of asparaginase for the immobilization on the O17-EC membrane was tested with 1.5 U, 5 U, and 10 U of asparaginase. The immobilization efficiency of asparaginase into

the L-Asn-sensing membrane was calculated based on Bradford method. The maximal reaction rate (V_{max}) and Michaelis-Menten constant (K_m) of the immobilized asparaginase were determined from the Lineweaver-Burk plot based on the ratio of the fluorescence intensities at λ_{em}=565 and 625 nm.

The reversibility of the L-Arg- and L-Asn-sensing membranes was examined at 0.1 and 0.5 mM L-Arg, and 0 and 5 mM L-Asn. The L-Arg-sensing membrane was first exposed to 0.1 mM L-Arg followed by 5 mM L-Arg, while the L-Asn-sensing membrane was exposed to 0.0 mM L-Asn followed by 5 mM L-Asn. The microplate reader was set for fluorescence measurements against time at an interval of 30 sec during measurements.

The effects of pH and temperature on the measurements of L-Arg and L-Asn were investigated. 1.0 mM L-Arg solutions in the pH range of 5.2-11.0 were exposed to the L-Arg-sensing membrane, whereas the L-Asn-sensing membrane was tested with 0.4 mM L-Asn in the pH range of 5.2-9.3. The L-Arg-sensing membranes were tested at different temperatures (25, 30, 33, 37, and 40 ^{o}C) at 1, 5, and 10 mM L-Arg, and the L-Asn-sensing membrane was tested at 21, 28, 31, 33, 36, and 40 ^{o}C at 5 mM L-Asn.

The storage stability of the L-Arg- and L-Asn-sensing membranes was evaluated with 0.1 mM L-Arg and 1 mM L-Asn by determining their repeatability by measuring the fluorescence intensity obtained initially and after a few hours and days.

The interfering effects of some components contained in blood and urine samples as well as in the fermentation processes on the L-Arg- and L-Asn-sensing membranes were investigated by using 0.5 and 7 mM L-Arg and 1 and 7 mM L-Asn as standard solutions. The effects of some amino acids and metabolites on the L-Arg- and L-Asn-sensing membranes were tested with 0.5 mM L-Arg and 1 mM L-Asn, while the effects of some saccharides, organic acids and salts were tested with 7 mM L-Arg and 7 mM L-Asn.

Artificial urine solution was prepared by mixing 2 mM citric acid, 1.1 mM lactic acid, 90 mM NaCl, 25 mM NH_4Cl, 0.4 mM uric acid, 7 mM creatinine, 0.25 mM $CaCl_2.2H_2O$, 2 mM $MgSO_4.7H_2O$, 25 mM $NaHCO_3$, 6 mM $NaNO_3$, 0.005 mM $FeSO_4$, 1.8 mM KH_2PO_4, 1.8 mM K_2HPO_4, and L-Arg or L-Asn in the concentration range of 0.1-2.0 mM at pH 7.2.

2.4 Ratiometric method

The ratiometric method for the L-Arg and L-Asn biosensors was based on the ratio of the fluorescence intensities (FI_{565} and FI_{625}) of the L-Arg-sensing membrane and the L-Asn-sensing membrane at an excitation wavelength of 460 nm (λ_{ex}= 460 nm) and two emission wavelengths (λ_{em}= 565 and 625 nm) as follows:

$$R = FI_{565} / FI_{625}$$

2.5 Data analysis

For repeated measurements of the L-Arg- and L-Asn-sensing membranes with different levels of pH, temperature, the differences in the fluorescence intensities were assessed by one-way analysis of variance (ANOVA). Significant differences between samples were accepted with p-value<0.05. Statistical tests were performed using the software InStat(vers.3.01, Graph Pad Software Inc., San Diego, USA).

3 Results and Discussion

3.1 Properties of the L-Arg- and L-Asn-sensing membranes

The properties of the fluorescent dye O17 and the O17-EC membrane are reported in our previous study [26]. The L-Arg- and L-Asn-sensing membranes included two layers; the below layer is the O17-EC membrane and the above layer is the membrane of the enzymes (arginase/ urease or asparaginase) immobilized into EC or EC-PU matrix. Ethyl cellulose (EC) was employed as a supporting material for both the layers so that the enzyme layer is strongly attached to the below layer after coating the mixture of ethyl cellulose and the enzymes onto the surface of the O17-EC membrane. AFM images showed the surface morphology of the O17-EC membrane, the L-Arg-sensing membrane and the L-Asn-sensing membrane (Supporting Information Fig.S1). That is, a smooth surface with a surface mean roughness (Ra) of 5.666 nm and a root mean square roughness (Rq) of 6.848 nm was observed with the O17-EC membrane, whereas the surfaces of the L-Arg- and L-Asn-sensing membranes had a higher roughness, i.e., the Ra of 18.334 nm and Rq of 27.274 nm for the L-Arg-sensing membrane and Ra of 6.088 nm and Rq of 8.769 nm for the L-Asn-sensing membrane. The increase in the roughness in the sensing membrane indicated successful immobilization of the enzymes into the EC or EC-PU matrix, layered over the O17-EC membrane.

The response time of the sensing membrane was not affected by the EC concentrations significantly. The thickness of the enzyme layer was not also a decisive factor affecting the response time of the sensing membrane. The response time is more related to the protein-capturing ability of the enzyme layer according to EC concentrations. Similar response times of the enzyme layer of 5% w/v EC and 10% w/v EC can attribute to the thickness and structure of the supporting material. That is, an enzyme

layer of 10% w/v EC is not too thick to hamper the reaction rate and its structure helps to immobilize more amount of the enzyme within the layer. The immobilization efficiency of both urease and arginase, on the second layer for the L-Arg-sensing membrane was very high at all the amounts of arginase used (Supporting Information Fig.S2). 94.4% of the two enzymes (arginase and 20 U of urease) were immobilized in the case of using 10 U of arginase, ~ 91% immobilized for 20 U of arginase used, 90% immobilized for 30 U of arginase used, 89% immobilized for 35 U of arginase used and 88% immobilized for 40 U of arginase used. The immobilization efficiency of urease (20 U) into the EC matrix was $\geq$ 95%. From the high immobilization efficiency of urease (20 U) the immobilization efficiency of only arginase into the EC matrix was estimated to be $\geq$ 85%. However, too large amounts of arginase immobilized into the EC matrix may hinder the NH_4^+ transport to contact with the O17-EC membrane. The high density of the enzyme supporting layer led to the difficult passage of NH_4^+ to react with the O17-EC membrane below the enzyme layer, whereas the lower amounts of arginase decreased the sensitivity of the O17-EC membrane. Thus, in our further experiments, 30 U of arginase and 20 U of urease were chosen to fabricate the L-Arg-sensing membrane based on the O17-EC membrane. In the case of asparaginase for the L-Asn-sensing membrane the immobilization efficiency of the enzyme on the second layer was very low at all the amounts of the enzyme used (Supporting Information Fig.S2). Only 8% of the asparaginase was immobilized in the case of using 5 U of asparaginase, ~10% of 10 U of asparaginase used and 15% of 1.5 U of asparaginase used. The low immobilization efficiency could be confirmed from low Ra and Rq values in the AFM image of the L-Asn-sensing membrane, as shown in Fig. 1. In this study, 1.5 U of asparaginase was used to fabricate the L-Asn-sensing membrane based on the O17-EC membrane..

3.2 Characterization of the L-Arg-sensing membrane

Fig. 1 shows the calibration curve of L-Arg calculated by the ratio of the two fluorescence intensities at λ_{em}=565 and 625 nm. The detection range of L-Arg based on the ratiometric calculation method could be divided into two linear ranges : 0.1-1.0 mM and 1.0-10 mM with high regression coefficient (r^2) values of $r^2_{0.1\text{-}1}$= 0.964 and $r^2_{1\text{-}10}$= 0.954. The LOD (S/N=3) was 0.082±0.0014 mM in the range of 0.1- 1.0 mM L-Arg.

Figure 1

According to the response of the O17-EC membrane to different ammonia concentrations in our previous research [26], the fluorescence intensity of the L-Arg-sensing membrane should increase at λ_{em}= 565 nm and decreased at λ_{em}= 625 nm with increasing concentrations of L-Arg. However, the fluorescence intensity at λ_{em}= 625 nm increased only slightly at low L-Arg concentrations. This could be attributed by only a low amount of ammonia reacted somewhat with the O17 dye in the following equation, meanwhile large amounts of dye were in a free state.

$$NH_4^+OH^- + H^+Dye^- \Leftrightarrow NH_4^+Dye^- + H_2O$$

The reaction mechanism of the O17 dye and ammonia produced from the catalytic reactions of L-Arg has been explained in our previous research [26]. Some modification of the original sensor could meet some unexpected matters, and in this case is the behaviour of the peak at λ_{em}= 625 nm which is not absolutely similar to the original behavior of the O17 dye. However, the ratiometric method can normalize the changes in the fluorescence intensities that are not related to the change in the target concentration. For example, the temporal and spatial distribution of the measured fluorescence intensity can typically fluctuate due to an unequal distribution of fluorophores within the sensor as well as to the variation in the dynamics of fluorophores in different media, etc. Therefore, the values

of the ratio of the fluorescence intensities at λ_{em}= 565 and 625 nm corresponded to L-Arg concentrations very well.

The K_m values of the arginase from different sources are as follows: 15.7 mM from *S.cerevisiae* [28] and *B. thuringiensis* [29], 13.5 mM from *B.subtilis* [30], 12.8 mM from *B.brevis* Nagano [31], 2.0 mM from bovine liver [32], and ~80 mM from plants [33]. Kinetic parameters of the arginase (30 U) and urease (20 U) co-immobilized on the supporting material of EC was evaluated via the Michaelis-Menten kinetics by using the ratio of two emission fluorescence intensities at λ_{em}=565 and 625 nm.

The V_{max}^{app} of 0.055 (mM min^{-1}) and K_m^{app} of 4.918 (mM) were obtained using the Hanes plot of the calibration data. However, the K_m^{app} value for the immobilized enzymes (arginase and urease) in the literature was 1.28±0.29 mM [21] and 2.2-2.27 mM [15]. The K_M is usually dependent upon the immobilization process and the supporting material and the lower K_m value indicates a greater affinity for L-Arg. The high K_m^{app} (4.918 mM) value in this study shows a low affinity of arginase and urease for L-Arg, which may be attributed to the conformation of the enzyme arrangement during the immobilization process. Slow reaction rate (V_{max}^{app}=0.055 mM min^{-1}) of the L-Arg-sensing membrane may result from the retardation of reaction product (NH_4^+OH) through a thick enzyme-immobilized layer to the O17-EC layer.

In general, the use of a large amount of an enzyme can increase the reaction rate and final products, increasing the sensitivity of sensor; however, the sensitivity of the sensor becomes rapidly limited to a narrow detection range due to reaching fast saturation of the hydrolysis reaction. It is clear that the hydrolysis reaction of L-Arg under the catalysis of arginase and urease occurred with amounts of the arginase and urease co-immobilized. The sensitivity of the L-Arg-sensing membrane is attributed to the effect of the supporting material (i.e. ethyl cellulose) on the transport of ammonia in solution reacting with the O17-EC membrane. The EC matrix did not release the ammonia produced from the

hydrolysis reaction of L-Arg rapidly nor slowly. Therefore, the response of the O17-EC membrane was still high at both low and high concentration ranges of L-Arg, however, the response time was long, t_{95}~4.8 min, as compared to other sensors [15,21]. The response time included the hydrolysis reaction time of L-Arg to produce urea and then ammonia as well as the transport time of ammonia passing through the enzyme-immobilized EC membrane to contact with the O17-EC membrane.

The reversibility of the L-Arg-sensing membrane could be expressed by the ratio of the fluorescence intensities at λ_{em}=565 and 625 nm (Supporting Information Fig.S3). The fluorescence intensities at both the emission wavelengths (λ_{em}=565 and 625 nm) had a low relative standard deviation (RSD), i.e. 1.7 % at 0.1 mM L-Arg and 2.9 % for 5 mM L-Arg at λ_{em}=565 nm and 2.1 % at 0.1 mM L-Arg and 3.6 % for 5 mM L-Arg at λ_{em}=625 nm.

Enzyme activity is mainly affected by pH and temperature. That is, the pH and temperature can increase or decrease the efficiency of a catalytic reaction in the enzyme-based biosensor. The arginase and urease co-immobilized into EC matrix preferred alkaline medium (i.e. pH range of 8.0-11.0) to weak acid medium (pH 5.0-7.0). The fluorescence intensity of the L-Arg-sensing membrane at 1.0 mM L-Arg did not change significantly (p-value=0.982 for λ_{em}=565 nm, p-value=0.975 for λ_{em}=625 nm) in the pH range of 5.2–8.2, whereas it increased considerably with increasing pH in the range of pH 8.9 to pH 11.0 (Supporting Information Fig.S4). These pH effects of L-Arg solution in the pH range of 5.2-8.2 are the same as those of ammonia solution [26]. Therefore, the L-Arg-sensing membrane was not influenced with ammonia produced from the hydrolysis reaction of L-Arg in the pH range of 5.2–8.2. In addition, alkaline medium is the favorite medium for these arginase and urease strains. Therefore, the response of the L-Arg-sensing membrane works well in alkaline medium via the ammonia produced from the hydrolysis reactions of L-Arg and urea or interferences from other alkaline factors. The L-Arg-sensing membrane at different temperatures does not seem to be considerably affected by temperature in the range of 25-40 ^{o}C at the L-Arg concentration in the range of 1.0-10 mM. (Supporting Information Fig.S4)

Ethyl cellulose (EC) was found to be a good supporting material for the fluorescent dye O17 as well as for enzyme immobilization. The layer of EC matrix prevented leakage of the enzymes, but it still created an open environment for the hydrolysis reaction of L-Arg and the sensing membrane of the O17 with ammonia as well. The storage stability of the L-Arg-sensing membrane was tested with 1.0 mM L-Arg. The ratio of the fluorescence intensities of 1.0 mM L-Arg at the initial use of the sensing membrane was set to 100%. Fig. 2 shows the relative percentage of the ratio decreased with increasing storage time, but remained to 41% after 20 days. That is, the L-Arg-sensing membrane including both a layer of O17-EC as a transducer and a layer of the EC matrix immobilized with two enzymes showed relatively good stability for 20 days.

Figure 2

The recovery capability of a biosensor suffers from some interferences existing in real samples. Blood or urine samples may contain many types of amino acids and metabolites. Some carbohydrates and salts can also be used in the fermentation processes for L-Arg. They can interfere the fluorescent L-Arg biosensor developed here. According to the results of one-way ANOVA with Tukey-Kramer multiple comparison post-test, there was no significant interference with some amino acids including creatine and creatinine. That is, the presence of some components such as L-histidine, L-glycine, L-phenylalanine, L-threonine, L-cysteine, creatine and creatinine or the mixture of these components did not affect the measurement of 0.5 mM L-Arg ($P>0.05$) of the L-Arg-sensing membrane significantly (Fig. 3). However, ammonium ion is a product in a sequence of the hydrolytic reactions of L-Arg. Ammonium ions of ammonium sulfite reacted with the O17-EC membrane and changed the fluorescence intensity of the L-Arg-sensing membrane. Therefore, the ratio of the fluorescence intensities (i.e. FI_{565}/FI_{625}) for 7 mM L-Arg in the presence of 30 mM ammonium sulfite

increased by 20%, compared to the ratio of the fluorescence intensities for 7 mM L-Arg without any additives, as shown in Fig. 3. But except ammonium ions of ammonium sulfite there was no significant interference with some components in the fermentation media. The L-Arg-sensing membrane was also stable after the fluorescence measurements of 7 mM L-Arg containing saccharides and salts.

Figure 3

The analytical performance of some fluorescence biosensors for L-Arg reproted in literature is compared to the ratiometric fluorescent biosensor in this study (Supporting Information Table S1). Colorimetric or fluorescent assays for L-Arg had high sensitivity and low linear detection range [34,35]. However, these assays are time-consuming and could not be selective regarding to interference of other amino acids and drugs found in biological fluids. Enzymes such as arginase, arginase deiminase and arginine decarboxylase are promising tools for quantitative determination of L-Arg, however, they should be immobilized onto several types of supporting materials such as polymer and electrode because of their high cost. Electrochemical enzyme-based sensors have some advantage owing to their operational simplicity, low expense of fabrication, high selectivity, and suitability for real-time detection, however, they also suffer from interferences and their sensitivity and stability need to be improved [21]. However, fluorescence sensors are more sensitive and specific for some analytes owing to direct measurement of their fluorophores. Ratiometric fluorescence technique offers an exact change in the fluorescence intensity of being independent of fluorophore concentration and intrinsic background fluorescence of the sensor. In addition, the fluorescent sensing membrane can be applied in a microtiter plate and used for high-throughput analysis of L-Arg. The

ratiometric fluorescent L-Arg biosensor developed in this study showed good linear detection range, i.e. from 0.1 to 1.0 mM and 1.0 to 10 mM L-Arg, short response time and relatively long storage time.

3.3 Characterization of the L-Asn-sensing membrane

Fig. 4 shows the linear detection range of L-Asn in the range of 0.1-2.0 mM with r^2 = 0.962 and an LOD of 0.074±0.0023 mM. The sensing membrane with only one enzyme usually seems to be more efficient than the sensing membrane with two enzymes in terms of sensitivity and response time. However, the response of the L-Asn-sensing membrane was not similar to the response of the O17-EC membrane to different ammonia concentrations in our previous research [26]. The fluorescence intensity of the L-Asn-sensing membrane increased at λ_{em}= 565 nm, but it was almost constant at λ_{em}= 625 nm with increasing L-Asn concentrations from 0 mM to 10 mM, because very little amount of ammonia probably produced during the catalytic hydrolysis reaction and reacted with the O17 dye.

Figure 4

The kinetic parameters, K_m and V_{max} were 0.303 mM and 0.547 μM min^{-1} for asparaginase from *Erwinia chrysanthemi* (Erwinase), respectively [39]. They were also 3.34 mM and 62.5 μM min^{-1} for asparaginase from the fruits of *Withanta somnifera*, whereas the parameters were 4.1 mM and 58.6 μM min^{-1} for the immobilized asparaginase, respectively. [40]. The K_m values were 120 mM for the asparaginase from *Escherichia coli* [24], 79 μM for recombinant asparaginase from *Erwinia carotovora* [25], and 80 μM at 37 °C and 5 μM at 70 °C for recombinant asparaginase from

Archaeoglobus fulgidus [23]. The kinetic parameters of the asparaginase immobilized in the matrix of EC and PU were calculated from the ratio of two fluorescence intensities at λ_{em}= 565 and 625 nm. The V_{max} of 1.186 mM min^{-1} and K_m of 22.05 mM were obtained from the Lineweaver-Burk plot. The high K_m value showed a low affinity of asparaginase for L-Asn. Faster reaction rate (V_{max}=1.186 mM min^{-1}) of the L-Asn-sensing membrane than that (V_{max}^{app}=0.055 mM min^{-1}) of the L-Arg-sensing membrane may result from the quick transport of reaction product (NH_4^+OH) through a thin enzyme-immobilized layer to the O17-EC layer. These results indicated that the hydrolysis reaction of L-Asn by the catalysis of asparaginase strongly occurred and decreased to the response time of the L-Asn-sensing membrane, that is, t_{95}= ~1-3 min at all the L-Asn concentrations.

Ammonia is produced directly through the hydrolysis of L-Asn. Therefore, the reproductivity of the L-Asn-sensing membrane was fast. This could be easily recognized when the L-Asn-sensing membrane was repeatedly exposed to 0 and 5.0 mM L-Asn, expressed by the ratio of the fluorescence intensities at λ_{em}=565 and 625 nm. (Supporting Information Fig.S5) The sensing membrane had low RSD of 2.1% for 0 mM L-Asn and 2.9% for 5 mM L-Asn .

The L-Asn-sensing membrane also preferred an alkaline medium (in the pH range of 8.0-9.0) to acidic medium (pH 5.0-7.0) (Supporting Information Fig.S6). At 0.4 mM L-Asn the ratio of the fluorescence intensities at λ_{em}=565 and 625 nm of the L-Asn-sensing membrane increased with increasing pH in the basic pH range. The L-Asn-sensing membrane was also stable in the temperature range of 28 ^oC to 40 ^oC at 0.1 mM L-Asn, however, the stability of the L-Asn-sensing membrane was lower at 21 ^oC than at 28 ^oC.

The storage stability of the L-Asn-sensing membrane was tested with 1.0 mM L-Asn . The ratio of the fluorescence intensities at 1.0 mM L-Asn was set as 100 % for the initial use of the sensing membrane. In Fig. 5 the relative percentage in the ratio of two fluorescence intensities increased with increasing storage time, because the fluorescence intensity at λ_{em}=625 nm decreased. The decrease in

the fluorescence intensity at λ_{em}=625 nm could be related to the versatile environment for enzyme catalysis and the response of the O17-EC membrane to ammonia produced.

Figure 5

Fig. 6 shows the effect of some components in blood and urine samples or in the fermentation processes on the L-Asn-sensing membrane. According to the results of one-way ANOVA with the Tukey-Kramer multiple comparison post-test, the measurement of 1.0 mM L-Asn in the presence of 1.0 mM L-asparate showed a significant difference with the standard sample (1.0 mM L-Asn, P=0.0031). However, the presence of other components such as L-glutamine, L-glycine, L-proline, L-alanine, L-serine, L-threonine and mixture of these components did not affect the measurement of 1.0 mM L-Asn (P>0.05). Some substrate and metabolites in the fermentation processes of glucose, fructose, urea, citrate and oxaloacetate increased the ratio of the fluorescence intensities (i.e. FI_{565}/FI_{625}) of the L-Asn-sensing membrane. This increase may result from the hydrolysis reaction of urea and ammonium sulfate in the sensing membrane, the change in the pH of sample after adding the additives, and also from the decrease in the fluorescence intensity at 630 mM. The interfering effects of the saccharides and organic acids can be overcome by controlling the pH value in the sample as well as diluting the sample.

Figure 6

Some biosensors have been developed for the detection of L-Asn and compared to that of the ratiometric fluorescent biosensor (Supporting Information Table S2). The analytical performance of the L-Asn biosensors is dependent on the detection technique and the enzyme (asparaginase) derived from bacteria, algae, fungi and plants. Colorimetric detection techniques for L-Asn are based on the formation of ammonia and its color change using the Nessler's reagent [25], whereas amperometric technique is based on the change in the potential of ammonium ion [23]. So far, there is no L-Asn biosensor based on the fluorescence technology. The ratiometric fluorescent L-Asn biosensor developed in this study also exhibited a good linear detection range (i.e. from 0.1 to 2.0 mM L-Asn), and short response time. Although the L-Asn-sensing membrane had a short storage time, many samples can be simultaneously analyzed through its immobilization into a 96-well microtiter plate.

3.4 Determination of L-Arg and L-Asn in artificial urine solution

The L-Arg- and L-Asn-sensing membranes were used to evaluate their analytical performance by determining the concentrations of L-Arg and L-Asn in artificial urine solution (AUS). Fig. 7 shows the measurement results of L-Arg or L-Asn concentrations in standard solution prepared in 10 mM PBS at pH 7.2 and also in AUS. The recovery percentage of the L-Arg- and L-Asn-sensing membranes was quite good (90-110%) in the L-Arg and L-Asn concentration range of 0.1-2.0 mM in AUS. It indicated that the L-Arg- and L-Asn-sensing membranes with the ratiometric method would be a powerful tool in clinical monitoring applications.

Figure 7

4 Concluding remarks

The ratiometric fluorescent biosensors for L-Arg and L-Asn were successfully fabricated using the sensing membranes consisting of immobilized enzymes and an ammonia-sensing layer, exhibiting high sensitivity, low limit of detection and good reversibility. The successful development of the ratiometric fluorescent biosensors will receive particular attention for the development of the high-throughput screening for analysis of L-Arg in biological fluids and drugs or L-Asn in leukaemia samples and in foods .

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Nomenclature

FI_{565}	[-]	fluorescence intensity at λ_{em}=565 nm
FI_{625}	[-]	fluorescence intensity at λ_{em}=625 nm
K_m	[mM]	Michaelis-Menten constant

K_m^{app}	[mM]	apparent Michaelis-Menten constant
Ra	[nm]	surface mean roughness
Rq	[nm]	root mean square roughness
RSD	[%]	relative standard deviation
r^2	[-]	regression coefficient
V_{max}	[mM min^{-1}]	maximal reaction rate
V_{max}^{app}	[mM min^{-1}]	apparent maximal reaction rate

Greek symbols

λ_{ex}	[nm]	excitation wavelength
λ_{em}	[nm]	emission wavelength

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Figure legends

Figure 1. (A) Fluorescence emission spectra of the L-Arg-sensing membrane to L-Arg in the concentrations range of 0.1-10 mM monitored at λ_{ex}= 460 nm, (B) Calibration curve of L-Arg calculated by the ratio of the two fluorescence intensities measured at λ_{em}=565 and 625 nm

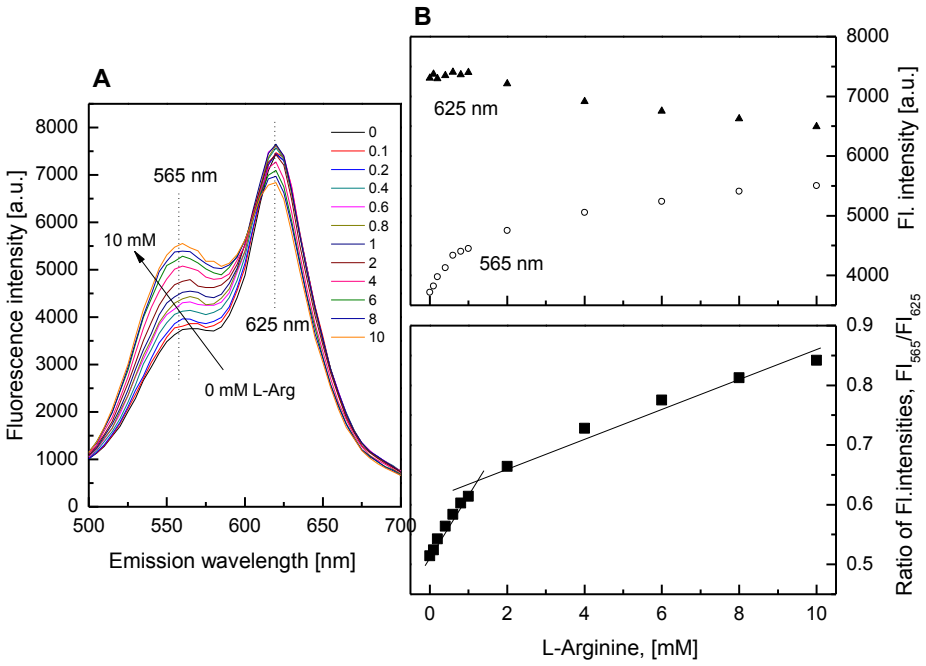

Figure 2. Storage stability of the L-Arg-sensing membrane at 1.0 mM L-Arg. The ratio of the two fluorescence intensities of 1.0 mM L-Arg at the initial use of the sensing membrane was set to 100%.

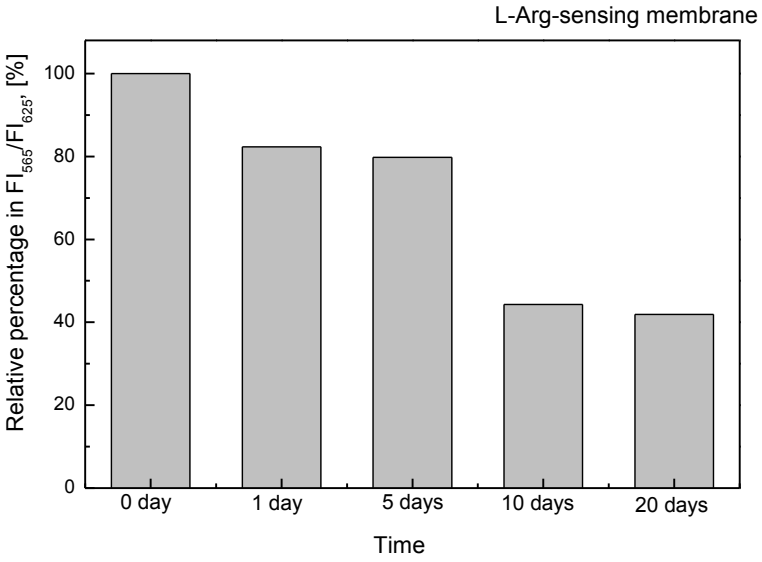

Figure 3. (A) Response of the L-Arg-sensing membrane in aqueous solutions of 7 mM L-Arg(Std7), 0 mM L-Arg(Std0), and each component (25 mM sucrose(Suc), 25 mM arabinose(Arb), 25 mM glucose(Glu), 30 mM $(NH_4)_2SO_4$(NSO), 1.5 mM KH_2PO_4(KPO), 1 mM $MgSO_4.7H_2O$ (MgSO), 0.1 mM $FeSO_4.7H_2O$(FeSO), 0.1 mM $MnSO_4.H_2O$(MnSO), 5 mM L-lysine(Lys) and mixture(Mix) of all these components) and (B) response of the L-Arg-sensing membrane for aqueous solutions of 0.5 mM L-Arg (Std) and 0.5 mM of each component (0.5 mM of creatine(Cr), creatinine(Crn), L-histidine(His), L-glycine(Gly), L-methionine(Met), L-aspartate(Asp), L-glutamine(Gln), L-cysteine(Cys), L-asparagine(Asn), L-threonine(Thr) and mixture(Mix) of all these components)

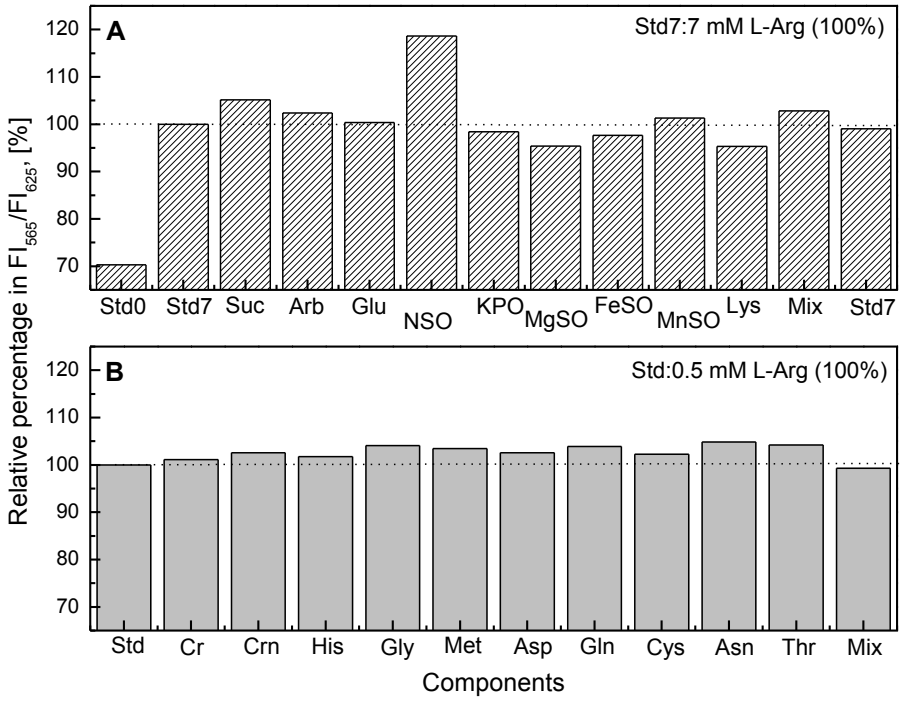

Figure 4. (A) Fluorescence emission spectra of the L-Asn-sensing membrane to L-Asn concentrations in the range of 0-10 mM monitored at λ_{ex}= 460 nm, (B) Calibration curve of L-Asn calculated by the ratio of the two fluorescence intensities at λ_{em}= 565 and 625 nm

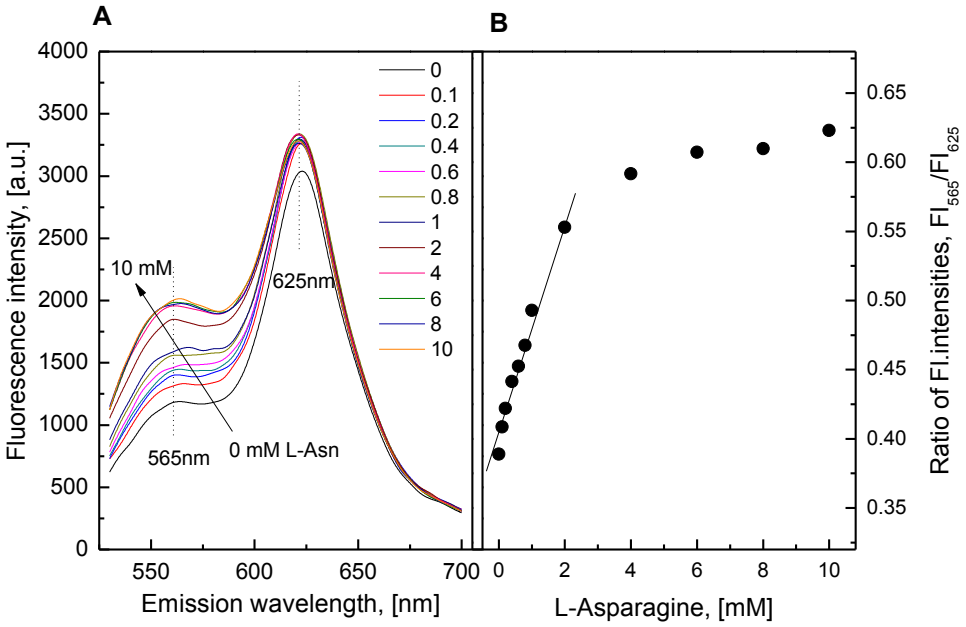

Figure 5. (A) Storage stability of the L-Asn-sensing membrane at 1 mM L-Asn (left), (B) two fluorescence emission spectra of the L-Asn-sensing membrane at λ_{ex}= 460 nm. The ratio of the two fluorescence intensities of 1.0 mM L-Asn at the initial use of the sensing membrane was set to 100 %.

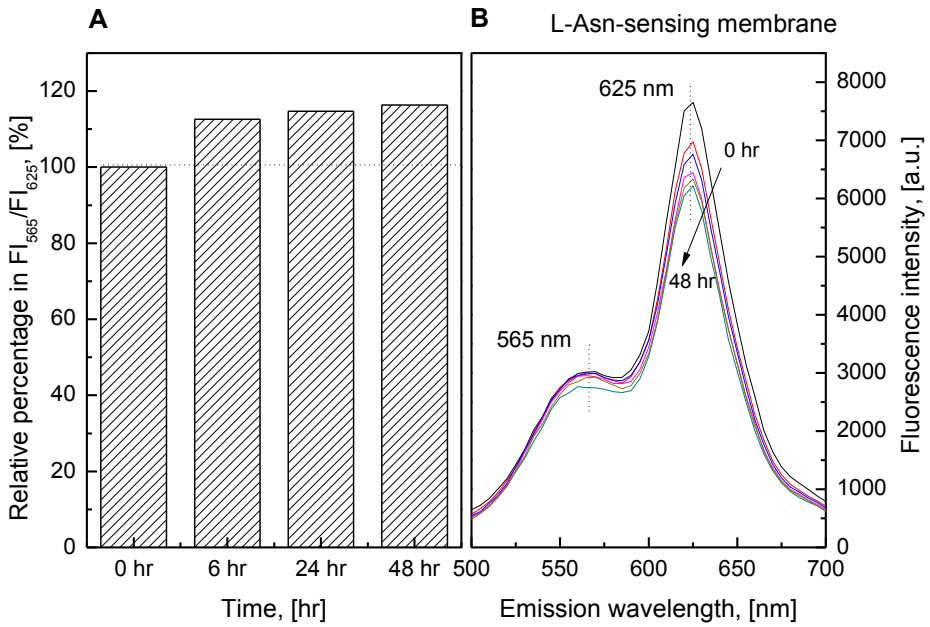

Figure 6. (A) Response of the L-Asn-sensing membrane for aqueous solutions of 7 mM L-Asn(Std7), 0 mM L-Asn(Std0) and each component (2 mM glycerol(Glr), 2 mM citrate(Cit), 10 mM glucose(Glc), 10 mM fructose(Fru), 7 mM oxaloacetate(Oxa), 2 mM urea, 30.3 mM ammonium sulfate(NHSO), and mixture(Mix) of all these components), and (B) response of the L-Asn-sensing membrane for aqueous solutions of 1.0 mM L-Asn (Std) and 1.0 mM of each component (1.0 mM of L-aspartate(Asp), L-glutamine(Gln), L-glycine(Gly), L-proline(Pro), L-alanine(Ala), L-serine(ser), L-threonine(Thr), and mixture(Mix) of all these components).

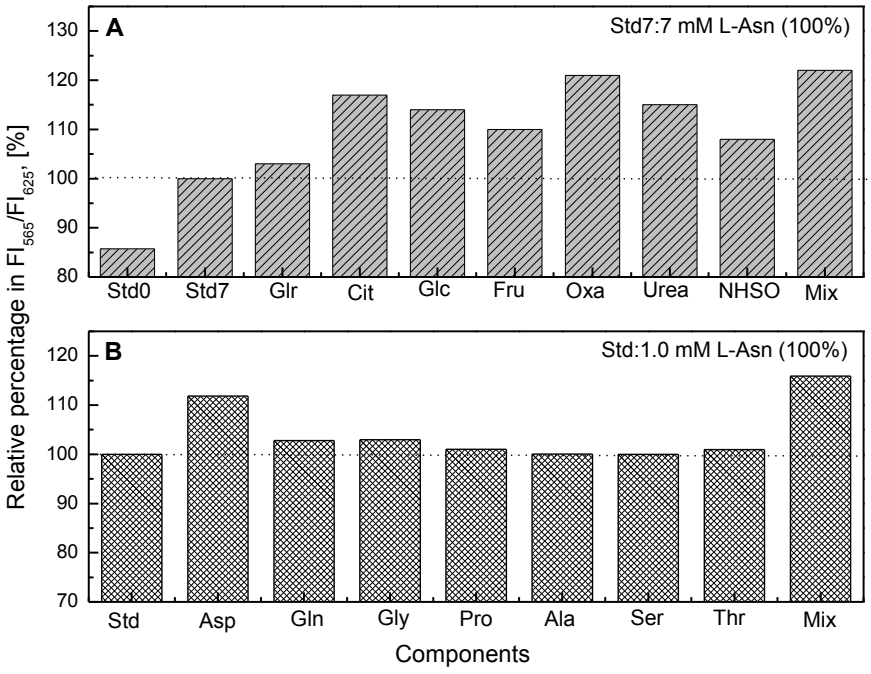

Figure 7. Comparison of L-Arg and L-Asn concentrations in standard solution and in artificial urine solution (AUS) measured with the L-Arg- and L-Asn-sensing membranes.