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In situ analysis and imaging of aromatic amidine at varying ligand density in solid phase

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Abstract

We report the development of a fast and accurate fluorescence-based assay for amidine linked to cellulose membranes and Sepharose gel. The assay is founded on the glyoxal reaction, which involves reaction of amidine group with glyoxal and an aromatic aldehyde, leading to the formation of a fluorophore that can be analyzed and quantified by fluorescence spectroscopy and imaging. While the assay has been reported previously for aromatic amidine estimation in solution phase, here we describe its adaptation and application to amidine linked to diverse forms of solid matrices, particularly benzamidine Sepharose and benzamidine-linked cellulose membranes. These functionalized porous matrices find important application in purification of serine proteases. The efficacy of a protein separation device is determined by, among other factors, the ligand (amidine) density. Hence, a sensitive and reproducible method for amidine quantitation in solid phase is needed. The glyoxal reaction was carried out on microbead-sized Sepharose gel and cellulose membranes. Calibration curves were developed for each phase, which established linearity in the range of 0–0.45 μmol per mL amidine for free amidine in solution, 0–0.45 μmol amidine per mL Sepharose gel, and 0–0.48 μmol per mL cellulose membrane. The assay showed high accuracy ($\sim 3.4\%$ error), precision ($\text{RSD} < 2\%$) and reproducibility. Finally, we show how this fluorescent labeling (glyoxal) method can provide a tool for imaging membranes and ligand distribution through confocal laser scanning microscopy.

Graphical abstract

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COMPLIANCE WITH ETHICAL STANDARDS

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Keywords

benzamidine; solid phase assay; glyoxal reaction; confocal laser scanning microscopy; ligand density

1. Introduction

Paper has been functionalized as a substrate for many applications in chromatography and point-of-care diagnostics[1–3]. Devices using paper-based chips have led to multiplex analyte assays[4] and sensors[5–7]. Cellulose paper is robust, environmentally friendly, biocompatible, biodegradable, cheap, and readily available. The cellulose hydroxyl groups (OH) can be functionalized with various molecules allowing the tuning of the surface properties and the development of bioassays or chromatography devices. For example, *para*-aminobenzamidine (pABA) is a known inhibitor of trypsin-type serine proteases and widely used as affinity ligand for purification of serine proteases through commercially available chromatographic matrices[8], adsorbers[9], and pABA cellulose membranes (PCM)[10]. Paper discs modified with pABA or other ligands may be used as sensors in bioreactors or diagnostic agents[11–14]. Myriad applications motivate the development of qualitative and quantitative methods for characterizing paper-based materials[15,16].

An important parameter for optimizing the binding of target molecule to separation matrix (e.g. Sepharose gel or cellulose membranes) is ligand concentration and distribution on the surface and within the matrix, i.e., ligand density[17,10,18–24]. A high-throughput method for quantifying ligand density on the matrix would hence allow rapid customization of the separation media for the desired application. However, the only reported method for amidine quantification in solid phase (Sepharose beads) involves acid digestion of the matrix, followed by HPLC-analysis[25]. The microscale fluorescence based method, developed in this study, provides a faster and economic tool for customization of amidine based separation media with high accuracy and precision.

Here, we report a method for amidine quantification on solid phase in a simple and fast fluorescence assay based on the known reaction[26] between benzamidine moiety and glyoxal group, followed by their reaction with benzaldehyde. This leads to the formation of 2-aryl-4-arylidine-2-imidazoline-5-one. The fluorophore has an excitation maximum at 320 nm and emission maximum at 490 nm in solution. The fluorophore was used by Fuller et al.

[27–29], for determination of different amidines in plasma and urine. Waalkes et al. used the glyoxal assay for determination of pentamidine in human plasma and urine and in mouse tissue[30].

Our work demonstrates that the glyoxal reaction, as has been reported in solution, can be adapted and applied to solid matrices for determination of pABA ligand density as well as qualitative analysis of ligand binding throughout the matrix volume. We describe the successful application of this method to fluorescent tagging of benzamidine moiety in two materials: pABA-linked cellulose membrane (PCM), and pABA Sepharose® gel (PSG). The method was used to estimate the ligand concentration of these analytes using a 96-well plate fluorometer, facilitating high-throughput screening of the amidine density. Moreover, the fluorescent properties of the fluorophore can be tailored by changing the aldehyde in the reaction. As an example, we show the tagging of PCM with fluorene-2-carboxaldehyde (FCB) via glyoxal reaction and its application to imaging PCM with confocal laser scanning microscopy (CLSM).

2. Experimental

2.1. Materials:

All chemicals were of analytical grade (Sigma-Aldrich, Inc., MO, USA) and used without further purification. Cellulose Acetate membranes (pore size 3.0 μm , diameter 50 mm, thickness 135 μm) were purchased from Sterlitech Corporation (WA, USA). Benzamidine-Sepharose® 4 Fast Flow beads were purchased from GE Healthcare Life Science (PA, USA). Deionized water was used for the preparation of all solutions.

2.2. Glyoxal assay.

2.2.1. Synthesis and characterization of 2-aryl-4-arylidine-2-imidazoline-5-one (AAIO): The fluorescent probe 2-aryl-4-arylidine-2-imidazoline-5-one (AAIO, Electronic Supplementary Material (ESM) Fig. S1, Compound 2) was synthesized starting from a solution of benzamidine hydrochloride (1 g, 6.38 mmol) and glyoxal-bisulfite (2 g, 7.51 mmol) in 42 mL of NaOH (1.0 N). The solution was left under magnetic stirring on an ice bath for 1 h. The pink-colored insoluble product was filtered under vacuum and extensively washed with cold water to obtain Compound 1 (2-phenyl-2-imidazolin-5-one) with a yield of 57.1% (0.589 g, 3.66 mmol). ^1H NMR (400 MHz, DMSO- d_6) δ 7.90 (m, 2H), 7.5 (m, 3H), δ 5.70 (s, 1H), δ 5.00 (s, 2H), FTIR (in cm^{-1}): 3284 (NH), 3170 (NH_2), 2890 ($=\text{CH}$ aromatic), 1613 ($\text{C}=\text{N}$), 1570 (NH_2 primary amine), 1439 and 1234 ($\text{C}-\text{N}$ stretch), 1180 and 1137 ($\text{C}-\text{OH}$ stretch), 1017 and 981 (benzene ring), and 766 (monosaturated ring).

All of the isolated product was dissolved in ethanol (25 mL), water (25 mL), and 10 N NaOH (5 mL) and reacted with benzaldehyde (0.760 mL, 7.45 mmol, 2.04 eq) for 48 h at room temperature. The resulting yellow crystalline solid was recovered by filtration and extensively washed with water to give 0.4475 g (1.80 mmol) of 2-aryl4-arylidine-2-imidazoline-5-one (**2**) (49.1% yield). ^1H NMR (400 MHz, DMSO- d_6) δ 8.30 (d, 2H), 8.20 (d, 2H), 7.65 (m, 3H), 7.50 (t, 2H), δ 7.45 (t, 2H), 7.05 (s, 1H) FTIR (in cm^{-1}): 3264

(secondary amine NH), 1626 (C=O), 1431 (CH₂), 1413 (primary amine C-N), 1260 (aromatic C-N), 901 and 883 (benzene ring), 773 and 686 (monosaturated ring).

2.2.2. Calibration curve for glyoxal reaction in solution phase: A calibration curve for estimating amidine in solution was established (Fig. 1a) by reacting 100 μ L of an aqueous solution of benzamidine hydrochloride at increasing concentration (0.085 – 1.75 mM) with 40.0 μ L of aqueous glyoxal-bisulfite (75 mM), and 50.0 μ L of benzaldehyde (784 mM) dissolved in absolute ethanol. The reaction was started upon the addition of 100 μ L of 10 N NaOH. The yield of this reaction was determined to be 75.8% by monitoring the decrease in absorbance of benzamidine at 254 nm. The excitation and emission spectra were obtained to determine the excitation and emission maxima respectively of the AAIO in solution (Table 1 and ESM Fig. S2) using the SpectraMax M3 Microplate Reader (Molecular Devices).

2.3. Quantification of amidine density in microbead based chromatography resin (Benzamidine Sepharose 4 Fast Flow[®]; GE Healthcare).

2.3.1. Calibration curve for glyoxal reaction in microbead sized chromatography resin: The calibration curve in microbead sized particles was prepared (Fig. 1a) using resin similar to Benzamidine Sepharose 4 Fast Flow[®] but without any pABA linked to it: Immobilized metal ion-affinity chromatography Sepharose Gel (GE Healthcare IMAC Sepharose[®] 6 Fast Flow). Standard solutions for glyoxal reaction (amidine concentration in the range of 0.05 – 1.00 mM) were prepared as described in section 2.2.2. Fifty μ L of the IMAC gel was pipetted in separate wells of a 96-well microplate and 50.0 μ L standard glyoxal reaction solution (0.05 – 1.00 mM amidine) added to each well resulting in Sepharose gel containing different amounts of the fluorophore AAIO. The excitation/emission maxima were determined for the AAIO fluorophore in this phase and finally fluorescence in each well measured using excitation and emission wavelengths of 330 nm and 510 nm, respectively (Table 1 and ESM Fig. S2).

2.3.2. Quantitative estimation of amidine in in pABA Sepharose 4 Fast Flow[®]: Fifty μ L of the Benzamidine Sepharose 4 Fast Flow[®] resin (ESM Fig. S3) were added to individual wells of a 96-well microplate and subjected to glyoxal reaction by adding 40.0 μ L of glyoxal-bisulfite (75 mM) solution and 50.0 μ L of benzaldehyde (784 mM) solution. NaOH solution (10N; 100 μ L) was then added dropwise. After allowing the reaction to take place for 45 min at room temperature, the fluorescence intensity in each well was measured using excitation wavelength of 350 nm and emission at 520 nm. The fluorescence intensity values were measured and converted to amidine concentration (μ mol/mL) using the calibration curve from IMAC Sepharose[®] gel (Fig. 1a).

2.4. Chemical modification of cellulose membranes.

The cellulose acetate membranes were deacetylated by allowing the membranes to react with a mixture of sodium hydroxide (1.0 N) and absolute ethanol (4:1 v/v) for 2 h at room temperature. The membranes were then washed with deionized water. Regenerated cellulose membranes thus obtained, were chemically modified following previously published protocol[10]. To obtain regenerated cellulose membranes with different spacer arm length

densities, membranes were reacted with varying quantities of epichlorohydrin (1.41 mmol (X), 2.81 mmol (2X), 5.63 mmol (4X), 9.28 mmol (6.6X), 14.06 mmol (10X), 18.71 mmol (13.3X), 28.13 mmol (20X), 70.32 mmol (50X), 140.65 mmol (100X), and 281.30 mmol (200X)) dissolved in a mixture of 19.0 mL of DMSO and 10.0 mL of NaOH (1.0 N). The membranes were washed with deionized water and suspended in a solution of *p*-aminobenzamidine dihydrochloride (2.92 mmol in 50.0 mL of 0.1 M phosphate buffer, pH 7.4). After incubation for 24 h at room temperature, the membranes were washed thoroughly with acidic deionized water (pH 2.0), followed by neutral deionized water to remove unbound pABA from membranes. Pieces from three different areas of membranes were cut, dried under vacuum, and analyzed through FT-IR spectroscopy. A Perkin Elmer Spectrum 100 FTIR spectrometer equipped with a Single reflection Diamond/ZnSe Standard Universal ATR Accessory was used to acquire 32 scans for each sample. This process resulted in membranes with different amidine densities, which will be referred to as X (1.41 mmol of epichlorohydrin) upto 200X (281.3 mmol of epichlorohydrin), depending upon the amount of epichlorohydrin added during the modification process as described above. The membrane modification scheme is depicted in Fig. 2a.

2.5. Quantification of amidine density in cellulose membranes.

2.5.1. Calibration curve for glyoxal reaction in membrane: The calibration curve was performed using the same method as described in section 2.3.1 for Sepharose gel, except that cellulose acetate membranes were used. The membranes were cut into uniform pieces using a paper hole cutter and each piece placed in a separate well of 96-well microplate (Fig. 1b). A volume of 3.0 μ L standard glyoxal reaction solution (0.05 – 1.00 mM amidine) was directly pipetted onto each membrane resulting in membrane pieces containing different amounts of the fluorophore AAIO. The excitation/emission maxima were determined for the AAIO fluorophore in this phase and then membranes were read for their fluorescence using the determined excitation emission maxima (λ_{Ex} = 360 nm, λ_{Em} = 500 nm).

2.5.2. Quantification of ligand density in pABA-modified cellulose membranes (PCMs): Parts of the PCM were cut into uniform pieces using paper hole cutter. The membrane pieces were placed in individual wells of a microplate and further reacted with 40.0 μ L of glyoxal-bisulfite (75 mM) solution and 50.0 μ L of benzaldehyde (784 mM) solution that were previously prepared and 100.0 μ L of 10 N NaOH added dropwise. The microplate containing membrane pieces was left on rocker for 45 min at room temperature. The liquid reaction mixture was withdrawn from each well and excitation and emission spectra run for the membranes to confirm the excitation and emission maxima, respectively. The fluorescence intensity of each membrane piece was then measured at 520 nm (λ_{Ex} = 370 nm). To quantify the amount of pABA in membranes, the fluorescence intensity value was converted to concentration (μ mol/mL; Fig. 3) using the membrane-based calibration curve. All the modified membranes were evaluated for their structural integrity using Scanning Electron Microscope (JEOL JSM-IT-100 with energy dispersive spectrometer; ESM Fig. S4).

2.6. Confocal Laser Scanning Microscopy (CLSM) analysis of the fluorescently labeled PCMs

2.6.1. Fluorescent labeling of PCMs with fluorene-2-carboxaldehyde

(FCB): PCMs were fluorescently labeled using the glyoxal method as described in section 2.5.2 except that benzaldehyde was substituted with FCB (Fig. 2a). The excitation and emission maxima were determined for the new fluorophore (2-aryl-4-9-fluorene-carboxaldehyde-2-imidazoline-5-one (AFIO)) on the membrane surface (λ_{Ex} = 490 nm λ_{Em} = 560 nm; Fig. 2b). The membranes were then submerged in aqueous Rhodamine B solution (1 mg/mL) (λ_{Ex} = 545 nm, λ_{Em} = 580 nm) for 10 min, washed three times with deionized water, and used for CLSM analysis.

2.6.2. CLSM microscope and parameter settings: An Olympus FLUOVIEW FV1000 inverted microscope with system version 1.7.3.0 (40x water objective (UApo/340); numerical aperture 1.15) was used in this study. Two lasers were used with transmissivities and wavelengths set at 10.0/488 nm (λ_{Ex} = 488 nm) for laser 1, and 4.0/543 nm for laser 2 (λ_{Ex} = 543 nm). Confocal aperture was set to automatic which equates to approximately 1 Airy disc. Filters were set to detect wavelengths of λ_{Em} = 504 nm in channel 1 and λ_{Em} = 567 nm in channel 2.

2.6.3. Z-stack analysis: Z-stacks consisting of x-y 1024 × 1024 voxel images were produced for each sample. Starting depth was set slightly above the membrane surface with z increasing with increasing depth starting from the near side.

3. Results and Discussion

This article describes the use of fluorescence-based methods to perform semi-quantitative *in situ* analysis of affinity ligand (amidine) density and distribution on a solid surface. The assay will be referred to as the glyoxal method and it is an adaptation of the method published by Jackson et al.[29], for quantitative estimation of aromatic amidine in solution. In this article we discuss its application for determining the concentration of an affinity ligand, *p*-aminobenzamidine (pABA) in two affinity separation devices: commercially available Benzamidine-Sepharose® 4 Fast Flow beads (GE Healthcare Life Science), and the cellulose affinity membranes synthesized in our laboratory.

3.1. Glyoxal method.

The goal of the assay being reported here is to quantify amidine moieties in pABA-modified membrane. Even though pABA is inherently fluorescent, the fluorescence is too weak, and its measurement is limited by short excitation and emission wavelengths[31]. This makes it nearly impossible to use its inherent fluorescence for the purpose of this study. The method being reported in this study is based on glyoxal reaction which involves reacting the benzamidine moiety with glyoxal, followed by aldol condensation with benzaldehyde (as described in ESM Fig. S1). This results in the formation of an imidazolone derivative (2-aryl-4-arylidine-2-imidazoline-5-one; (ESM Fig. S1; Compound 2) that is fluorescent (λ_{Ex} = 320 nm λ_{Em} = 490 nm) (Table 1 and ESM Fig. S2a). The standard curve was developed using benzamidine instead of pABA as the standard amidine group, to prevent interference

from the *para*-amino group in forming the fluorophore (Fig. 1). The method was established to be linear in the range of 0 – 0.45 μmol per mL. At higher concentrations a saturation effect was observed. The excitation and emission maxima can shift depending on the solvent used (data not shown). A shift was also observed in both excitation and emission maxima depending on whether the reacting amidine group was in solution, linked to microbeads, or to cellulose membranes. The excitation/emission curves for glyoxal reaction product in different media have been presented in ESM Fig. S2a.

Performing the glyoxal method on a solid surface, such as regenerated cellulose membrane that has amidine group covalently linked to it, should lead to formation of the imidazolone derivative fluorophore on the substrate. Quantitation of this fluorophore and hence the amidine groups is possible through direct measurement of the fluorescence intensity on solid surface. However, the challenge lies in translating the fluorescence values into actual amidine concentrations in an accurate and precise manner. The following two sections demonstrate a successful creation of standard plots using amidine linked to two different solid surfaces.

3.2. Quantitative estimation of amidine immobilized on micron-sized bead structures.

With the aim of testing and validating the glyoxal method for estimation of amidine group in solid phase, we chose to apply it to the determination of ligand concentration in the commercially available chromatography matrix, Benzamidine-Sepharose 4 Fast Flow[®] (GE Healthcare Life Science, ESM Fig. S3)[32]. The product is sold as a viscous suspension of micron-sized porous beads.

To plot a standard curve, a similar microsphere-based gel matrix lacking amidine group, IMAC Sepharose 6 Fast Flow[®] gel (GE Healthcare Life Science), was chosen. Known quantities of glyoxal reaction mixture consisting of amidine in the range of 0–0.040 μmoles were pipetted onto fixed quantities of gel suspension in 96-well microplate. The fluorophore formed in the glyoxal reaction is expected to diffuse uniformly through the gel suspension. As noted for liquid standards before, a saturation effect was observed in this case too at amidine concentrations higher than 0.45 μmol per mL Sepharose gel (Fig. 1a). A fluorometric measurement of these suspensions instead of the liquid reaction mixture by itself, permits accounting for the effect of the solid media on the fluorescence intensity. It was observed that λ_{Ex} shifted from 320 nm to 330 nm while λ_{Em} shifted from 490 nm to 510 nm when the glyoxal reaction product was entrapped in the microbead suspension (Table 1 and ESM Fig. S2). Fluorescence measurements were thus carried out accordingly.

The subsequent step was to perform the glyoxal reaction on PSG and use the calibration curve obtained with IMAC Sepharose to convert the fluorescence intensity values into amidine concentrations. In case of PSG, a slight red-shift was observed in the emission maximum (520 nm) due to the *para*-amino group in the aromatic ring (Table 1 and ESM Fig. S2). The results from this analysis gave an average ligand density of 11.59 $\mu\text{mol/mL}$ of the Benzamidine-Sepharose[®] gel which was in close agreement with the manufacturer's specification (12 μmol benzamidine/mL medium)[32]. The standard deviations, as calculated from measurements performed in triplicate, were within a range of 0.25 – 1.83 $\mu\text{mol/mL}$. The method used by the manufacturer to quantify amidine density involves acid

hydrolysis of the Benzamidine-Sepharose gel followed by HPLC ion-pair chromatography coupled with photometric detection of benzamidine[25]. This method is tedious and disruptive while its sensitivity is equivalent to the glyoxal method reported here. These findings validate the glyoxal method for amidine quantification in solid phase. We thus proceeded to apply this method for determination of amidine ligand density on the cellulose membranes that were developed in our laboratory for protein separation.

3.3. Quantitative estimation of amidine immobilized on cellulose membrane.

The affinity membranes were prepared using a method developed previously in our laboratory[10]. Briefly, cellulose acetate membranes (3.0 μm pore size; *Sterlitech*) were deacetylated in a basic ethanol/water solution. The regenerated cellulose membrane was then reacted with epichlorohydrin that forms a five-atom spacer arm on membrane surface, followed by linking to pABA as the affinity ligand (Fig. 2a). The resulting membrane is an affinity separation device that will capture serine proteases through its terminal amidine group. By varying the amount of spacer arm (epichlorohydrin) added to the reaction, it is possible to control the ligand density on the membranes.

A calibration curve was developed in the membrane phase by first preparing a series of glyoxal reaction mixtures containing benzamidine in the range of 0–1.0 mM, and then pipetting a fixed volume (3.0 μL) of each concentration reaction mixture onto individual punch holes (average diameter 5.0 mm, thickness 135 μm) of unmodified cellulose acetate membranes. The excitation and emission spectra showed λ_{Ex} at 360 nm and λ_{Em} at 500 nm (Table 1 and ESM Fig. S2). A sketch of the protocol developed for the calibration curve is shown in Fig. 1b. The volume pipetted onto the membrane piece spread uniformly through the punch hole membrane. The standard curve thus obtained (Fig. 1a) is linear in the range 0–0.48 $\mu\text{mol/mL}$ membrane, with a linear correlation coefficient (R^2) of 0.99 and RSD up to 2.0%.

As mentioned previously, affinity membranes of different ligand (amidine) densities were prepared by reacting the RC membranes with varying amounts of epichlorohydrin (spacer arm) relative to the amidine concentration. The reference amount of epichlorohydrin (1.41 mmol) is being referred to as X. The resulting membranes with varying amounts of spacer arm (up to 200X) will lead to proportional amounts of amidine group linkage to the membrane. As a consequence, membranes are obtained with different amounts of amidine immobilized throughout the membrane volume. When the pABA linked membranes were subjected to glyoxal reaction, λ_{Ex} and λ_{Em} shifted to 370 and 520 nm respectively (Table 1 and ESM Fig. S2), predictably due to the presence of the amino group as previously discussed. The first indication of the effectiveness of the assay was seen in the appearance of different intensities of yellow color on the membranes carrying different amount of pABA. Fluorometric measurements revealed that the membranes treated with increasing amounts of epichlorohydrin yielded a trend of increasing fluorescence intensity until a certain point, after which a saturation effect was seen (Fig. 3). This saturation might be a consequence of unavailability of free hydroxyl groups on membrane surface for linking to epichlorohydrin and then to benzamidine. The saturation effect is reached around 100X which corresponds to a ligand density of roughly 0.4 μmoles amidine per cm^3 of membrane. The relative standard

deviation for different membrane samples tested was up to 9%. This deviation is higher as compared to the standard samples due to batch-to-batch variation in yields from the chemical modification process of the membranes, along with reasons described later with regard to the inherent structure of the membrane. When the ligand density is lower than 4X, partial degradation of the membranes was observed visually and later confirmed by SEM microscopy (ESM Fig. S4). Experiments are currently being carried out in our laboratory using these membranes to study the effect of ligand density on protein binding capacity. Preliminary results show that ligand density affects the amount of protein that binds to the membrane irrespective of protein size (Data not included).

3.4. Confocal Laser Scanning microscope (CLSM) analysis of the fluorescently labeled PCM.

3.4.1. Fluorescent labeling of pABA-linked cellulose membrane: Substituting benzaldehyde with fluorene-2-carboxaldehyde in the glyoxal reaction still resulted in the formation of a fluorophore but with a different spectroscopic profile ($\lambda_{Ex} = 490$ nm and $\lambda_{Em} = 560$ nm) (Fig. 2b). While this new probe allowed for a quantitative estimation of amidine in the same way as the benzaldehyde based probe, the resultant fluorophore allows for a range of analyses at different wavelengths. In this section we describe the use of the new probe for imaging using CLSM. Similar studies on membranes reported in the past use either fluorescently labeled protein[33,34] or a fluorescent dye adsorbed to the matrix[35–37] or both[38,39]. To our knowledge this is the first report of *in situ* analysis and imaging of ligand itself on a separation device.

3.4.2. CLSM analysis: Fig. 4 shows the tagged pABA and rhodamine (RH) channels of the CLSM images of membranes functionalized with pABA (ligand density 10X). The images show a profile of the membrane structure that combines a regular fiber-mesh structure with larger secondary channel-like cavities similar to what was observed in SEM images (ESM Fig. S4). Regions with fiber mesh membrane structure are visible in both channels and absence of significant single-color (green or red) regions in the composite suggests colocalization of both fluorophores (voxels where both fluorophores are present) on the membrane and uniform pABA distribution throughout membrane volume[40]. The 2D density plot (Fig. 4d) shows an elongated ellipsoid form confirming a high degree of colocalization and a very small number of voxels with predominantly RH content[40] (significantly less than 5% of the voxels). The fact that most points fall in a very small area of the plot means that the colors and red-to-green rates of most voxels in the image are very similar. Colocalization of pABA and Rhodamine in the sample can be inferred by observing that most points are close to their linear fit (blue line). The ligand density of the membrane used in Figure 4 is 10X. Other ligand densities show a similar 2D density plot.

The effect of attenuation in the z-stack was found to be similar in all membrane samples (ESM Fig. S5). The average signal intensity curve of a z-stack of an attenuating medium as the depth z increases can be modeled by a stochastic interpretation of the Beer-Lambert law, an exponential decay pattern given by $N_{tr} = N_i \exp(-\beta z)$, where $\beta = \sigma c$, σ is the extinction cross section per obstacle (dependent on the material) and c is the obstacle concentration[41]. Its derivation assumes statistical homogeneity, allowing for patches and

voids throughout the layer. Data analysis (ESM Fig. S5) confirmed a good fit with the Beer-Lambert attenuation model. This is consistent with a uniform penetration of pABA and RH into the membrane up to depths of 30 μm , the maximum depth probed in our samples using CLSM.

3.4.3. Three-dimensional (3D) images with attenuation correction: The raw images were processed to compensate for attenuation with depth[42]. As mentioned above, the z-level with maximum average brightness was selected as the first depth that falls completely inside the membrane. Using this as the base image I_0 , images I_z corresponding to deeper X-Y scans were modified using

$$\hat{I}_z(x, y, c) = \sqrt{\frac{\sigma_{0,c}^2}{\sigma_{z,c}^2}} \left(I_z(x, y, c) - \mu_{z,c} \right) + \mu_{0,c}$$

where $I_z(x, y, c)$ represents the intensity of the pixel x, y in channel c at depth z , $\mu_{z,c}$ and $\sigma_{z,c}^2$ are the average intensity and intensity variance of the pixels in channel c at depth z , respectively. After this transformation all the images in the z-stack will have the same average intensity and intensity variance. This corrects the brightness and sharpness[43] while preserving the shapes of the intensity histograms unlike more sophisticated methods such as histogram warping[44].

After data processing, a 3D representation based on the CLSM, z-stack was produced using the *3D Viewer* plugin[45] of the *Fiji* distribution[46] of *ImageJ* software[47] (Fig. 5). It shows a portion of the membrane prepared with epichlorohydrin 50X dilution. Uniform color at all sampled depths is consistent with uniform functionalization of the membrane.

4. Conclusion

Trypsin-type serine protease are pharmaceutically and industrially very important enzymes. The *in vitro* production processes for these enzymes require downstream processing which involves use of benzamidine linked separation media. As pointed out before, ligand density is an important determinant of the protein binding capacity of these separation matrices. As a means to facilitate rapid optimization of amidine density on separation matrices, a rapid and precise fluorescence assay for (aromatic) amidine quantification in solid phase was developed and reported in this study. This method not only allows the determination of benzamidine ligand density in commonly used protein purification matrices but also enables imaging of these matrices with CLSM. Optimization of the assay was carried out in liquid phase as well as in two solid matrices linked to benzamidine as affinity ligand. It was shown that the glyoxal reaction so developed can be applied to amidine estimation in solid phase. The method has practical advantages such as the use of cheap and commercially available reactants (approximately 7 USD per gram of reagents or 1.8 USD/1,000 assays) and minimal non-toxic solvents. It is fast (25 min of incubation at room temperature) and does not depend on chemical digestion of the matrix unlike standard HPLC or elemental analysis. Quantification of the ligand density of pABA-linked cellulose membranes was performed

with good precision. It was also demonstrated that the glyoxal reaction can be carried out with different aldehydes leading to formation of new fluorophores. As an example, we show the use of FCB for imaging membranes with confocal laser scanning microscopy. This imaging modality confirmed a uniform distribution of benzamidine at different depths of the membranes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1a.

Standard curves for the glyoxal reaction in different media using benzamidine as the standard. Linearity was observed in the range of 0–0.45 μmol per mL benzamidine in solution phase; 0–0.48 μmol per mL in cellulose acetate membrane; and 0–0.45 μmol per mL when the glyoxal reaction product was present in IMAC Sepharose® gel

Fig. 1b.

Scheme for preparation of glyoxal reaction standard plot in cellulose membranes

Fig. 2a.

(Steps 1–3) Chemical functionalization of regenerated cellulose membranes with 5-atom spacer arms and coupling of pABA; (Step 4a) Fluorescent labeling of amidine on the membrane surface using glyoxal method; (Step 4b) Fluorescent labeling of pABA via glyoxal reaction using fluorene-2-carboxaldehyde

Fig. 2b.
Excitation/emission spectra of glyoxal reaction product (2-aryl-4-9-fluorencarboxaldehyde-2-imidazoline-5-one (AFIO)) linked to the membranes

Fig. 3.
Ligand density in pABA membranes chemically modified with varying amounts of epichlorohydrin as determined by performing glyoxal method directly on the membranes.

Fig. 4. CLSM images of a cellulose membrane functionalized with pABA and the corresponding density plot. Images (a) and (b) represent the fluorescently labeled pABA and Rhodamine channels respectively. Image (c) represents the overlap of the pABA (green) and RH (red) channels. The 2D density plot (d) represents a scatter plot where there is a point with coordinates $(/g/, /r/)$ for each voxel with green color intensity $/g/$ and red intensity $/r/$. Shaded regions, from darkest to lightest, contain 50%, 25%, 20% and 5% of the points respectively.

Fig. 5.

Three-dimensional rendering of the membrane functionalized with pABA tagged with fluorene-2-carboxaldehyde and stained with RH. The composite image (a) represents the RH channel as red and the pABA channel as green. Regions of colocalization result in a yellowish color. Yellow color at all observed depths in the composite A indicates uniform distribution of pABA. Composites (b) and (c) were created manually by removing a rectangular region of the glyoxal and RH channels, respectively, to show how RH-only and pABA-only regions would look. The width of the sample portion is 65.7 μm

Table 1.

Effect of media on the excitation and emission maxima of AAIO

Glyoxal reaction product (AAIO)	Excitation maxima (nm) λ_{Ex}	Emission maxima (nm) λ_{Em}
in solution	320	490
suspended in IMAC Sepharose® gel	330	510
pABA-Sepharose FF®	350	520
adsorbed to cellulose acetate membrane	360	500
pABA linked RC membrane	370	520