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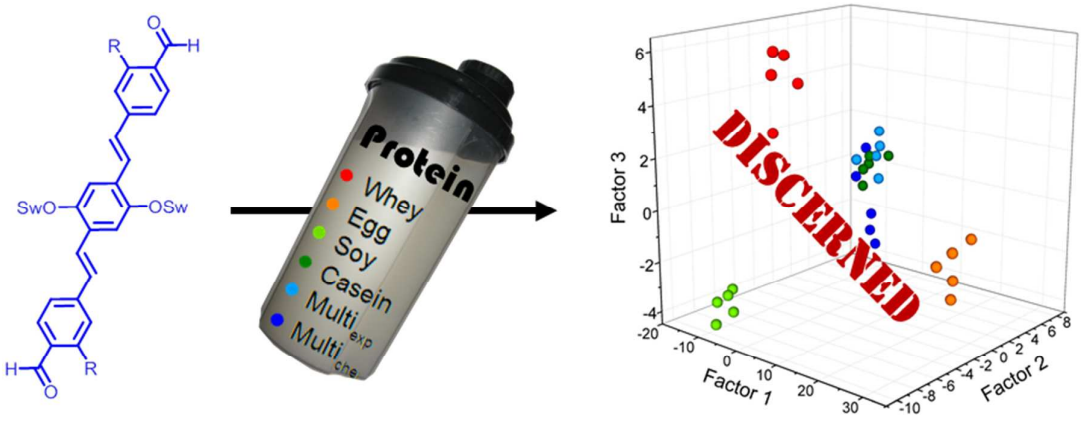

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ARTICLE

Distyrylbenzene-aldehydes: identification of proteins in water

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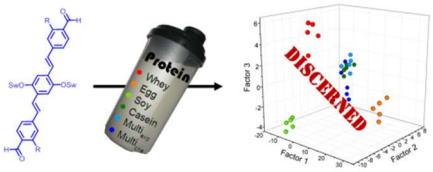
Three different, water soluble, aldehyde-appended distyrylbenzene (DSB) derivatives were prepared. Their interaction with different albumin variants (human, porcine, bovine, lactalbumin, ovalbumin) is investigated (pH 11). All three fluorophores exhibit graded, protein-dependent fluorescence turn-on at slightly differing wavelengths. Linear discriminant analysis (LDA) differentiated all of the investigated albumins and was used to discern commercially available protein shakes. The three DSB derivatives barely react with the constituting amino acids but cysteine. In the proteins significant fluorescence signals are generated, probably due to a combination of imine/N,S-aminal formation and hydrophobic interactions.



Introduction

Herein we describe the discrimination of different albumins using fluorescence changes in a simple three-compound library and apply this system in the discrimination of protein shake powders.

Detection, discrimination and identification of proteins is important in biomedical applications to identify disease states, inflammation factors etc.[1] Advanced solutions for this problem include mass spectrometry, antibody cascades and enzyme essays but also more conventional approaches such as 2D-electrophoresis or affinity chromatography. The enzyme-linked immunosorbent assay (ELISA)[2] as the most extensively used method for discrimination of proteins is based on a "lock-key" system using antibodies. Application of this technique demands expanded preliminary tests to obtain specific antibody-antigen pairs.[3] In serum proteomics the combination of reversed phase liquid chromatography (RPLC) with mass spectrometric devices is a high throughput solution with major impact.[4] These bioanalytical tools, while sophisticated, are cost-intensive and also in most cases unsuitable if one does not have access to an extensive instrument park. Recently, an alternative approach using fluorophores or chromophores has sprung up. Here, instead of looking for specific responses or signals obtained by investigation of colour or emission wavelength and intensity changes, data fields are created, which allow the differential identification of chemical or biochemical species using fingerprint-type approaches. Elegant examples are Suslick's[5] colorimetric sensors for the fingerprinting of volatile organic compounds (VOCs), Anslyn's[6] replacement assays and Walt's[7] fluorescent microspheres, to name important concepts.[8] A powerful fluorescence sensor for proteins (and other biological entities including bacteria and eukaryotic cells) was developed by Rotello et al.,[9] in which cationic, monolayer-protected gold nanoparticle quench the fluorescence of water soluble anionic conjugated polymers; upon addition of the analyte the complex is disrupted and fluorescence turn-on results – an analogue data space that identifies almost any bioanalyte, as long as one can create a fingerprint from an authentic sample. Looking at continuous fluorescence changes upon exposure to an analyte is

followed by execution of a linear discriminant analysis (LDA) of the fluorescence intensities. LDA has also been used successfully by Lavigne et al. for spoilage of fish, detecting amines by a water soluble polythiophene derivative.[10] In most of these cases, binding of the analyte to the indicator is achieved by electrostatic, van der Waals type and other weak intermolecular forces. However, these define only a small part of response options. A challenging task for such sensor arrays is to discern structurally related analytes, i.e. the members of a family of protein. Catalytic nanomaterials like MgO and BaO were applied to fingerprint serum albumins through thermochemiluminescence (TCL).[11] Furthermore Fan et. al. recently reported a dicyanomethylene-*4H*-chromene based probe able to discriminate HSA from BSA by selective site I binding inducing a distinct fluorescence response.[12] We show here a response system, which must combine a chemical reaction, i.e. covalent binding with weak interactions in the fluorescence turn-on sensing of serum albumins, and as a real-life testbed the identification of powdered protein shakes.

Materials and methods

Reagents and Proteins

All reagents and proteins were of analytical reagent grade and have been purchased from Sigma Aldrich (Germany). Buffers were purchased from VWR (Germany): pH 7 (KH_2PO_4/Na_2HPO_4), pH 9 (H_3BO_3/NaOH/KCl), or from Sigma Aldrich: pH 11 (H_3BO_3/NaOH/KCl), pH 13 (glycine/NaOH/NaCl). For synthetic procedures and corresponding analytics please check the supporting information.

Photographs

Buffered solutions of the fluorophores (c = 4.4 µM, V = 8 mL) were prepared in glass vials and 2 mg of the protein targets were added. Photographs were taken after 1 h reaction time under UV-light irradiation (λ = 365 nm) in darkness using a Canon EOS 7D camera equipped with a Canon EF-S 66 mm objective. Fixed settings of the camera: (JPEG format, shutter speed 0.10 s, ISO value 100, aperture F2.8, white balance 6500 K and Adobe RGB 1986 color space).

UV-VIS and fluorescence measurements

The assay solutions used for the photographs were further diluted with buffer solution by a factor of 3 for UV-VIS and fluorescence measurements. Absorption spectra were recorded on a Jasco UV-VIS V-660 spectrophotometer and fluorescence measurements were carried out on a Jasco FP-6500 spectrofluorometer using rectangular quartz cuvettes (10 x 10 x 40 mm).

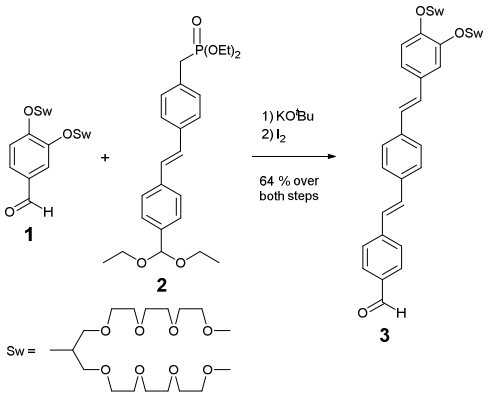
Linear discriminant analysis (LDA)

Fluorescence measurements for LDA were performed after 1 h reaction time of buffered aqueous solutions (pH 11, c = 4.4 µM) of **3**, **4** and **10** with albumins or protein shakes. The final concentrations were A = 0.038 at 280 nm, which was calibrated using UV-VIS spectroscopy and achieved by dilution with buffer. The fluorescence intensity values at 495 nm (albumins) and at 465 nm (protein shakes) were recorded with excitation at 380 nm. This process was repeated for each protein target to generate five replicates. Thus, the five albumins (or six protein shakes) were tested against a three fluorophore array (**3**, **4** and **10**) five times to afford a data matrix of 3 fluorophores x 5 albumins (or 6 protein shakes) x 5 replicates. To obtain a fluorescence reference value, the pure buffered fluorophore solution was measured at A_{280} = 0.038 and its response subtracted from the fluorescence response in presence of analytes. The data matrices were processed using classical LDA in SYSTAT (version 13.0). In LDA, all variables were used in the model (complete mode) and the tolerance was set as 0.001. Fluorescence response patterns were transformed to canonical patterns. The Mahalanobis distances of each individual pattern to the centroid of each group in a multidimensional space were calculated and the assignment of the case was based on the shortest Mahalanobis distance.

Results and discussion

Synthesis of water-soluble distyrylbenzene (DSB) aldehydes

We have recently prepared the distyrylbenzene (DSB) derivative **4** (Fig. 1) as a water soluble, amine-reactive fluorophore, which, to our disappointment, was non-reactive in the detection of amino acids. Only cysteine gave fluorescence turn-on in water through the formation of an N,S-aminal. For this reason we synthesized the derivatives **3** and **10** with different electronic properties. The synthesis of compounds such as **3**, **4** and **10** is modular and involves Heck or Horner type chemistry. Horner-reaction of **1** (Scheme 1) with the protected phosphonate **2** furnishes **3** in 64% yield after deprotection.



Scheme 1 Synthesis of monoaldehyde **3** in a Horner reaction.

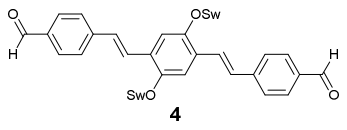
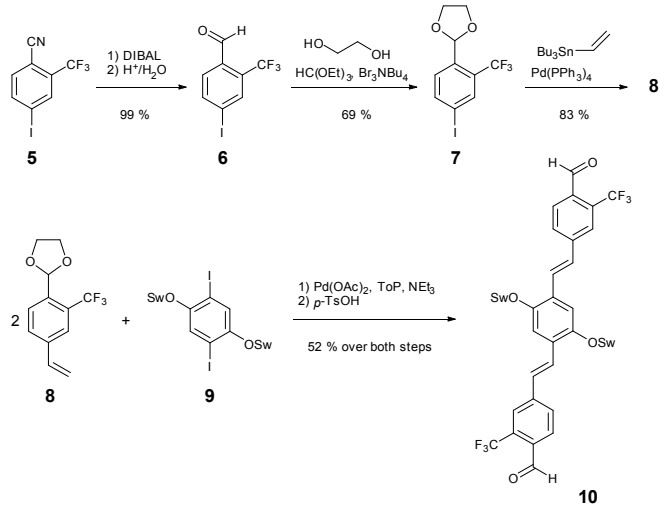


Fig. 1 Structure of dialdehyde **4**.

The synthesis of **10** is a bit more complicated (Scheme 2). Starting from **5**, reduction, protection and Stille coupling gives the protected building block **8**, which is Heck-coupled to the diiodide **9** furnishes the target molecule **10** after deprotection (52%).



Scheme 2 Synthesis of **10** by a sequence of a Stille and a Heck coupling.

All three DSB-derivatives, **3**, **4**, and **10** are stable, yellow, viscous oils and - due to attachment of branched oligo ethylene glycol side chains (Swallowtails, Sw) - well soluble in water, where they are almost non-fluorescent. The lack of fluorescence is explained by the stabilization of the n-π* state, which then is deactivated radiationless.

Interaction of aldehyde distyrylbenzenes (DSBs) with proteins

Upon reaction with simple amines, fluorescent imines form, making this system useful as amine sensor in water.[13] However, amino acids, even lysine or arginine, did not give a good response. Only cysteine formed a brightly fluorescent N,S-aminal with **4**. Also, the amines only react with the dialdehyde at a pH >10, testament to the acid lability of the formed imines. Is such a system useful for the detection of proteins?

We have presumed covalent interaction of the aldehyde groups with side chains of proteins, e.g. cysteins resulting in formation of N,S-aminals at elevated pH. To base our assumption on a experiment we further exposed a DSB without aldehyde moieties to the protein targets and could not observe changes in emission. Also, our dialdehydes **4** and **10** might work similar to an extended glutaraldehyde, crosslinking two or more protein chains.

pH-dependent protein sensing studies

In a first experiment (Fig. 2) we exposed buffered aqueous solutions (pH 7, 9, 11, 13) of **3**, **4** and **10** towards seven different proteins. At pH 7 there is no change in fluorescence upon addition of the proteins. At pH 9 some of the proteins induce fluorescence turn-on and at pH 13 all of them, with the exception of cytochrome c, induce fluorescence.

Interestingly enough, all of the seven proteins can be discriminated by a simple photographic technique. The pH-dependent color changes in fluorescence can be considered as an additional discriminating factor. In Fig. 3 the emission spectra of the three DSBs at pH 11 in the presence and in the absence of the proteins are shown. The discrimination is, of course, also possible using spectroscopic data. BSA invariably shows the largest fluorescence turn-on, followed by histone, both known as cysteine rich proteins. To determine the limit of detection for the model analyte BSA we exposed fluorophore solutions (c = 4.4 µM, pH 11) to different concentrations of the protein. The fluorescence turn-on is already quite distinct for BSA at a concentration of 25 mg/L (0.38 µmol/L), qualifying our approach for detection in serum.[14] For all applied DSBs the fluorescence response is almost complete at a protein concentration of 250 mg/L (Fig. S4). At pH 11 or 13 the proteins are not in their native state anymore, but probably denatured and unfolded. BSA exhibits 35 cysteine residues, which can react with the aldehyde groups of the DSBs under thioaminal etc. formation (Table 1).

Fig. 4 though shows convincingly that proteins and amino acids show fundamentally different reactivites to **3**, **4** and **10**. Amino acids react only weakly towards the DSBs and only cysteine induces some fluorescence turn-on.

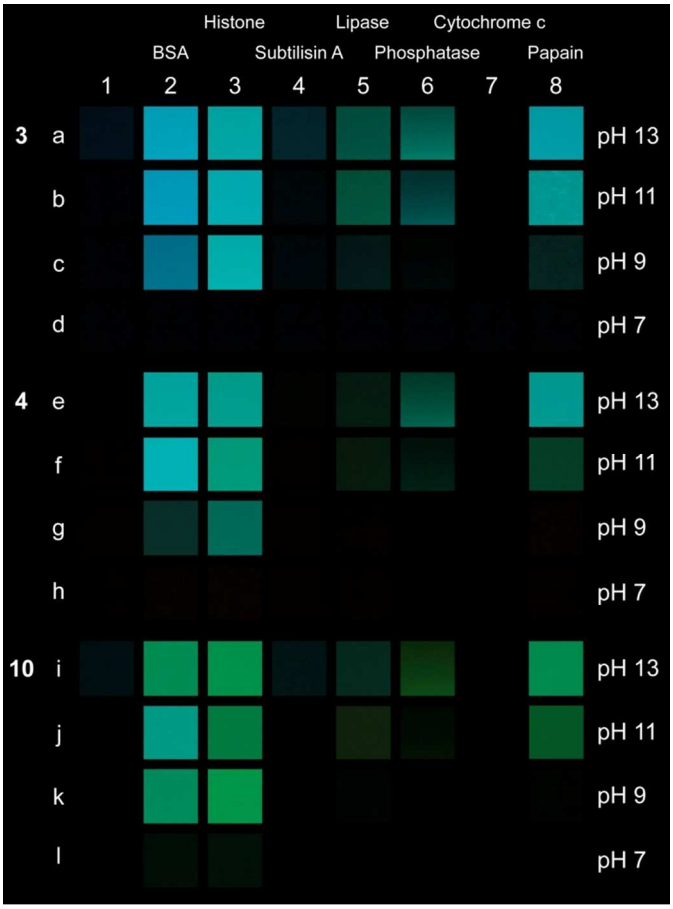


Fig. 2 Photographs of buffered aqueous solutions (c = 4.4 µM) of **3**, **4** and **10** upon addition of proteins 2-8 (left to right, c = 0.25 g/L). Buffers: pH 13

(glycine/NaOH/NaCl) (a, e, i), pH 11 (H_3BO_3/NaOH/KCl) (b, f, j), pH 9 (H_3BO_3/NaOH/KCl) (c, g, k), pH 7 (KH_2PO_4/Na_2HPO_4) (d, h, l). Columns: (1) fluorophore reference, (2) albumin from bovine serum (BSA), (3) histone from calf thymus, (4) subtilisin A, (5) lipase, (6) acid phosphatase from potato, (7) cytochrome c, (8) papain from papaya.

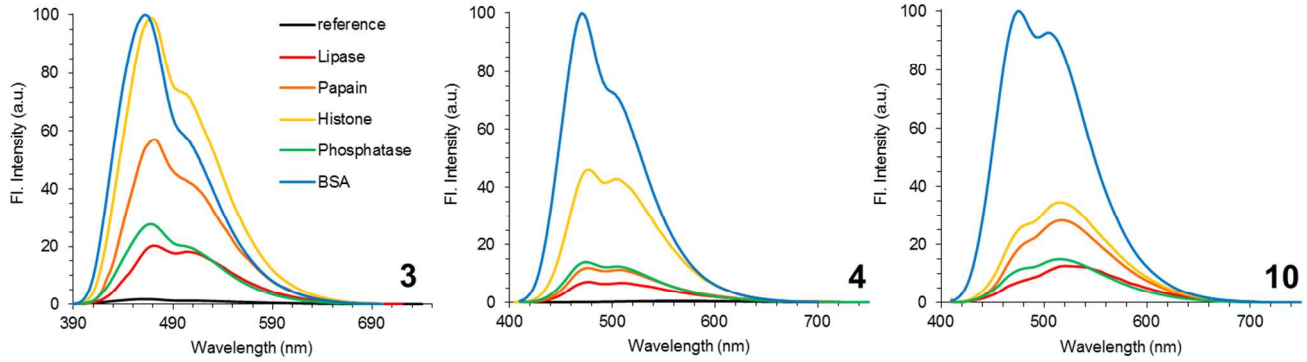


Fig. 3 Non-normalized emission spectra of buffered aqueous solutions (pH 11) of **3** (left), **4** (middle) and **10** (right) upon addition of different proteins.

Table 1 Properties of the proteins used as sensing targets

Protein	MW/kDa	pI
BSA	66.4	4.8
Cytochrome c	12.3	10.7
Histone	21.5	10.8
HSA	66.5	4.7
Lactalbumin	14.2	4.5
Lipase	58.0	5.6
Ovalbumin	44.3	4.9
Papain	23.0	9.6
Acid Phosphatase	110	5.2
PSA	66.4	4.8
Subtilisin A	30.3	9.4

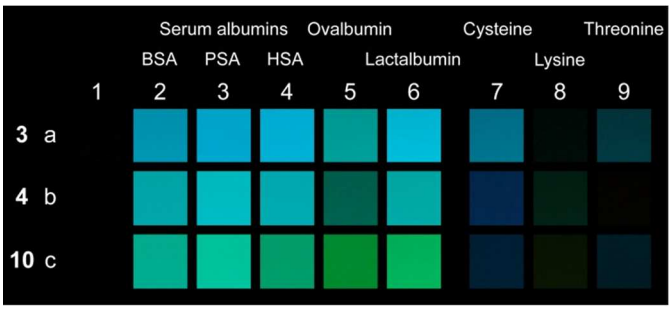


Fig. 4 Photographs of buffered aqueous solutions (pH 11, c = 4.4 µM) of **3**, **4** and **10** upon addition of albumins 2-6 (left to right, c = 0.25 g/L). Buffer: pH 11. Columns: (1) fluorophore reference, (2) albumin from bovine serum (BSA), (3) albumin from porcine serum (PSA), (4) albumin from human serum (HSA), (5) ovalbumin, (6) lactalbumin in comparison with amino acids 7-9. Columns: (7) cysteine, (8) lysine, (9) threonine.

Discrimination inbetween a family of proteins

Are members of the albumin family discerned? Reaction of bovine, porcine and human serum albumin as well as ovalbumin and lactalbumin with **3**, **4**, and **10** at pH 11 leads to turn-on in all five cases. BSA, PSA, HSA and lactalbumin are similar and only show subtle differences in their fluorescence response, while ovalbumin invokes a significant difference in its fluorescence response as put in record by photography (Fig. 4). While the serum albumins are all around 600 amino acids long, ovalbumin consists of 386 and lactalbumin only 142 amino acids. The percentage of hydrophobic residues in these proteins is for BSA 36.9%, HSA 38.6%, PSA 38.0%, lactalbumin 39.4% and for ovalbumin 44.8%.

The variance in the amount of hydrophobic side chains is small, and probably does not play a great role in the denatured state. The amount of cysteines is perhaps more interesting. In bovine serum albumin (BSA), porcine serum albumin (PSA) and human serum albumin (HSA) 5.8 % of the amino acids are cysteines. In lactalbumin the percentage is 5.6%, while in ovalbumin only 3.5% of all amino acid monomers are cysteine units. If one looks at the response colour of the DSBs towards ovalbumin, a distinct green tint is visible. We speculate that in the absence of sufficient numbers of cysteine units, lysine will form an imine, which has a red-shifted emission from the blue emitting thioaminals, as all of the conjugation between the DSB and the carbonyl unit is pinched off. Fig. 5 shows the non-normalized emission spectra that belong to the experiments documented in Fig. 4. Here also both red shift and decrease of brightness of the fluorescence are observed when comparing the reaction of the DSBs towards the serum albumins and ovalbumin.

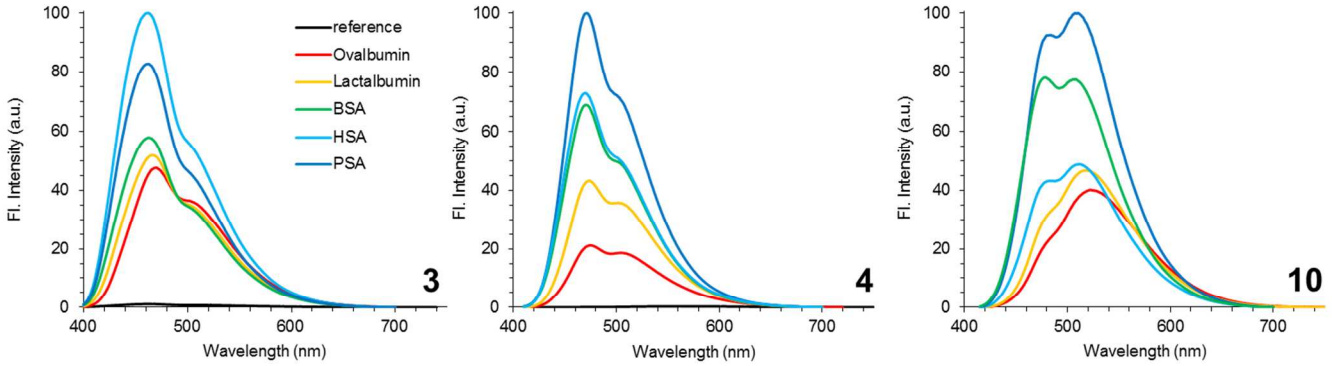


Fig. 5 Non-normalized emission spectra of buffered aqueous solutions (pH 11) of **3** (left), **4** (middle) and **10** (right) upon addition of different albumins.

Linear discriminant analysis (LDA) of albumins

For a more quantitative treatment, LDA discerns all of the albumins. After 1 h reaction time of **3**, **4** and **10** with the albumins, the fluorescence response was recorded. The respective combinations display similar absorption and emission spectra, allowing the same excitation (380 nm) and emission wavelength (495 nm). Concentration was calibrated to a standard UV absorbance (A_{280} = 0.038) to generate a training matrix (3 DSBs x 5 albumins x 5 replicates, Table S1). The response of the pure fluorophore solution in buffer was measured at A_{280} = 0.038 and subtracted from the fluorescence responses in presence of analytes (Fig. 6).

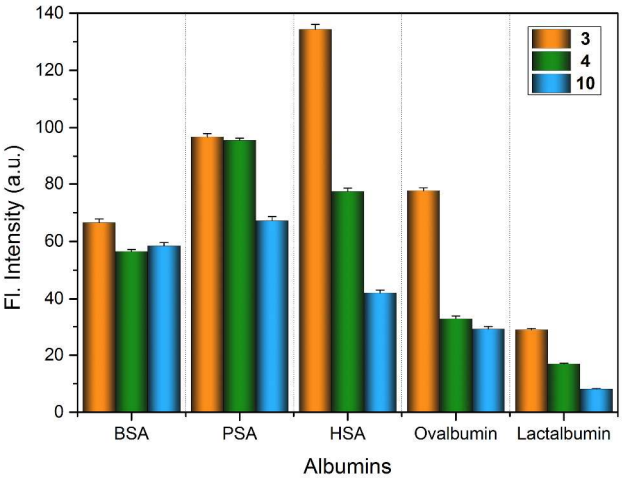


Fig. 6 Fluorescence intensity of the three DSB array (**3**, **4** and **10**) against five albumin analytes (A_{280} = 0.038) as an average of five parallel measurements.

The fluorescence response patterns in the training matrix were transformed into canonical factors, clustered into five distinct groups (one for each albumin analyte) as visualized in the canonical scores plot (Fig. 7). The 25 training cases (5 albumins x 5 replicates) are properly assigned to their respective group, resulting in a 100% accuracy.

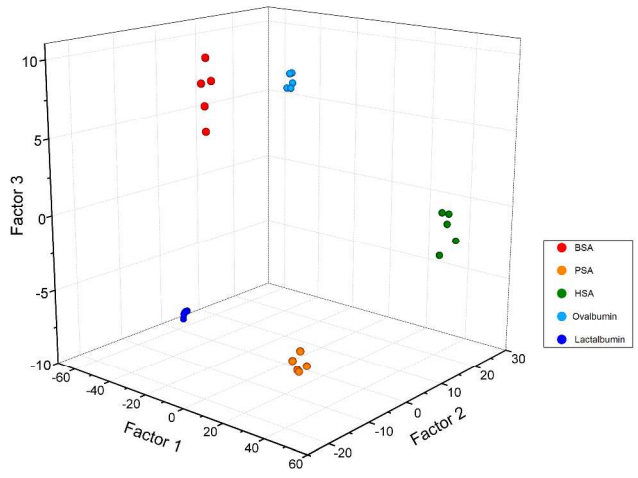


Fig. 7 Canonical scores plot for the first three factors of simplified fluorescence response patterns obtained with an array of **3**, **4**, and **10** against five albumin analytes (A_{280} = 0.038).

In a blind test another 18 unknown albumin samples were subjected to analysis *via* LDA. The new cases were classified to the groups generated through the training matrix according to their Mahalanobis distances. All were correctly assigned, resulting in an identification accuracy of 100%. Thus, reproducibility and suitability of our DSB array in detection and identification even within a family of proteins are confirmed.

Discrimination of protein shakes as a real-life testbed

A useful application for protein discrimination should be if we could discern complex matrices. We investigated protein shakes as easily available testbeds. We selected a whey-based, an egg-based, a soy-based, a casein-based and two multicomponent mixtures, of which one was expensive and the other a budget one. Fig. 8 shows the photographs taken after the exposure of the three DSBs to the six different protein shakes. The photographs look similar, only subtle differences can be gleaned.

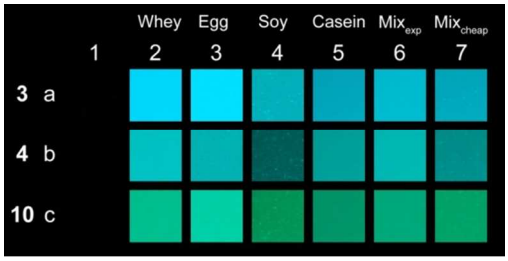


Fig.8 Photographs of buffered aqueous solutions (pH 11, c = 4.4 μM) of **3**, **4** and **10** upon addition of protein shakes 2-7 (left to right, c = 0.25 g/L). Buffer: pH 11. Columns: (1) fluorophore reference, (2) whey protein, (3) egg protein, (4) soy protein, (5) casein, (6) expensive multicomponent shake, (7) cheap multicomponent shake.

The emission spectra (Fig. 9) are a bit more instructive but here also mostly only fluorescence turn-on occurs. Whey protein is a mix of lactalbumin (ca. 25%), lactoglobulin (ca. 65%) and BSA (ca. 8%), casein is a mix of several proteins (αS1-, αS2-, β-, κ-casein), while soy protein is a mix of different legume globulins; egg protein is composed of ovalbumin (ca. 54%), ovotransferrin (ca. 12%), ovomucins (ca. 11 %) and ovoglobulins (ca. 8 %). While the changes in spectroscopic properties are only subtle (Fig. 9), the canonical scores plot (Fig. 10) is instructive. Protein shakes based on soy, egg and whey are easily discerned. However, the cheap, the expensive and the casein-based protein shakes all cluster together. The main ingredient of multi component protein shakes is the-easy-to-isolate milk protein, which consist up to 80% of casein. We assume therefore that the mixed protein shakes contain mainly casein, and that the low-price and the expensive protein shakes are very similar but not identical.

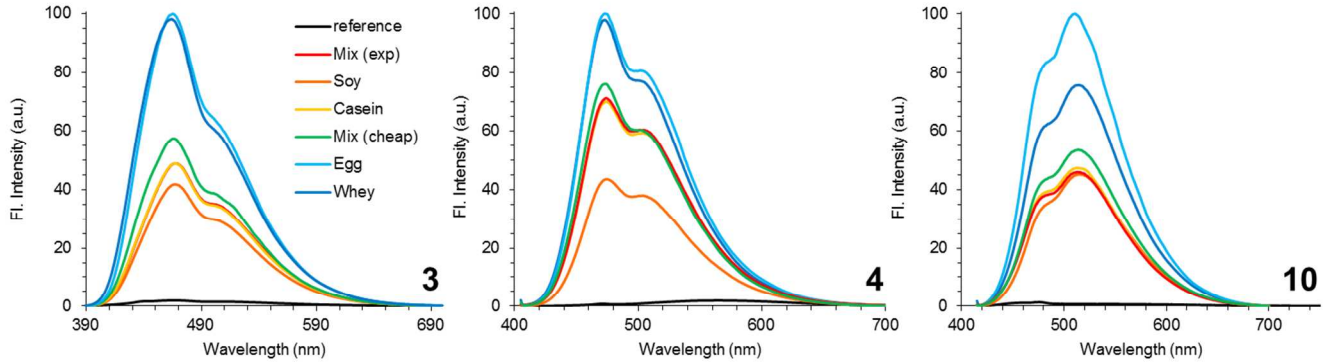


Fig. 9 Non-normalized emission spectra of buffered aqueous solutions (pH 11) of **3** (left), **4** (middle) and **10** (right) upon addition of different protein shakes.

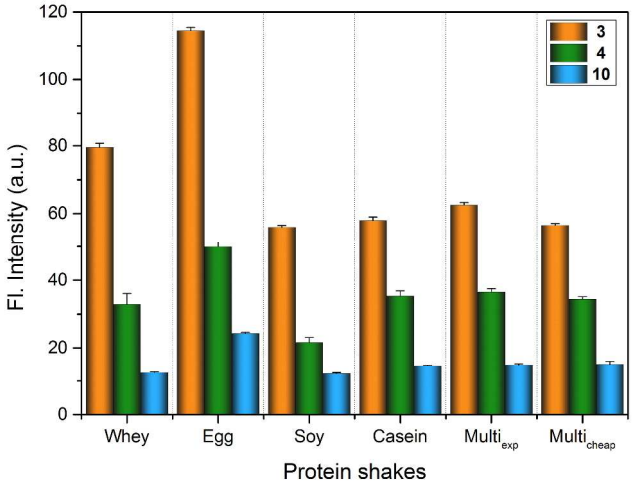


Fig. 10 Fluorescence intensity of the three DSB array (**3**, **4** and **10**) against six proteinshake analytes (A_{280} = 0.038) as an average of five parallel measurements.

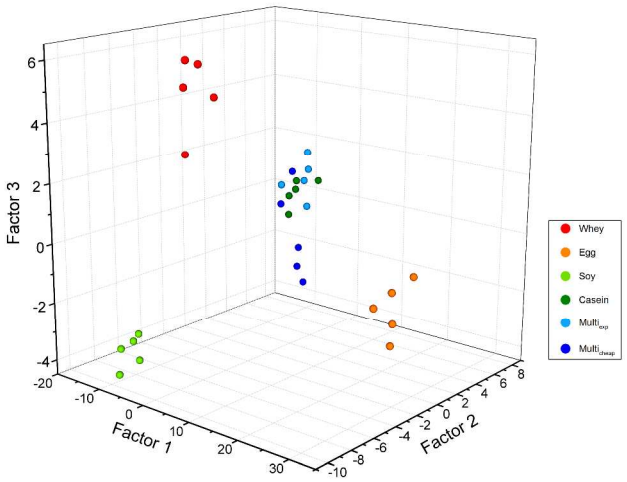


Fig. 11 Canonical scores plot for the first three factors of simplified fluorescence response patterns obtained with an array of **3**, **4**, and **10** against six protein shake analytes (A_{280} = 0.038), quintuple experiments.

Conclusions

Three aldehyde-substituted DSB-derivatives react with albumins in water at elevated pH. All of the albumins cause fluorescence turn-on but to a different degree with respect to their cysteine content. LDA cleanly discerns different albumins. In all cases fluorescence turn-on was observed - modulated by the chemical nature of the proteins. The turn-on of the DSBs' fluorescence was due to a combination of thioaminal or imine formation and generalized hydrophobic interactions. The constituent amino acids do not give a strong turn-on, so the protein chain must have a protecting influence on the DSB fluorophore. The fluorophores also differentiated commercially available protein shakes, using LDA of the recorded emission spectra. The selectivity of this small sensor set is surprising and powerful despite the absence of specific binding, multivalency or other auxiliary effects and can be considered as a cost-effective, easy to handle alternative to well established approaches. In future we will study the interaction of differently substituted DSBs - all easily synthesized - with proteins to enhance selectivity, signal intensity and width of application. Attractive but challenging targets would be protein imbalances in human serum or also detection of specific analytes in serum. To tune the versatility of the DSBs we can attach positive or negative charge and/or incorporate aldehyde-DSBs into conjugated polymers. We foresee a bright future for DSB-based biosensory and quality-control applications.

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Notes and references

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† Footnotes should appear here. These might include comments relevant to but not central to the matter under discussion, limited experimental and spectral data, and crystallographic data.

Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/b000000x/

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