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Bovine Serum Albumin Bioconjugation with FITC

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Abstract This laboratory provides students a basic approach to bioconjugation experiments, a topic which is not typically covered in didactical laboratories. Students are required to characterize a fluorescent probe as well as a protein and then to perform a bioconjugate experiment. The resulting marked protein is characterized by means of UV-Vis spectroscopy.

Keywords: *bioconjugation, bovine serum Albumin, FITC, UV-Vis spectroscopy*

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1. Introduction

Bioconjugation [1] consists in the linking of two or more molecules forming a novel complex with combined properties of its individual starting components. Thus, a protein can be labelled to another molecule capable of being detected to form a traceable conjugate. The resulting complex is thus visible, detectable and can be easily localized through various techniques (fluorescence spectroscopy, imaging), or directly used for measurements. Growing interest is caused by the possibility of applying a variety of bioconjugate techniques in biological, medicinal, polymer, material science or chemistry fields.

One of the key component for biological research and medicinal chemistry relies in the ability to produce a labelled protein with specificity for another target molecule. Proteins are therefore the most common targets for modification or conjugation techniques. Serum albumins, in particular, are the most extensively studied and applied proteins because of its availability, low cost, stability and unusual ligand-binding properties. [2] For this reason, a huge number of papers and reviews dealing with albumins have been published so far [2,3,4,5] and above all for its low cost has been used for several laboratory experiments [6,7,8,9].

Proteins, nucleic acids and other molecules can be labelled by small modifying agents, referred as tags and probes. They usually contain atomic or molecular moieties with a sensitive detectability due to intrinsic chemical or atomic property such as fluorescence, radioactivity or bioaffinity toward another protein. A probe can contain a reactive group able to link to the functional groups of the biomolecule of interest. After the modification of a protein, the probe is covalently attached. In this way, the protein results permanently labelled with a unique detectable property.

Fluorescent tags [1] can provide very high sensitivity exploiting their large quantum emission yield. There are a great number of fluorophores available for a wide range of applications. Each fluorophore has a chromogenic property and different reactive groups (for example amine-reactive or thiol-reactive) which can couple to specific functional groups of the target molecules. Fluorescein is one of the most popular among fluorescent labelling agents. In particularly, fluorescein isothiocyanate (FITC, Figure 1) is one of the most popular fluorescent probe. Its fluorescent character is due to its three-ring planar structure.

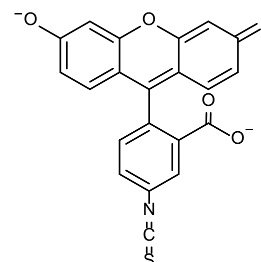

Figure 1. Structure of fluorescein isothiocyanate (FITC)

Isothiocyanates are reactive toward nucleophilic sites such as amines (i.e. lysines), sulfhydryls (i.e. cysteines) and the phenolate ion of tyrosine side chains [10]. However, FITC is highly selective for N-terminal amines in proteins [11] because it is able to form stable products only with primary amine groups. The reaction proceeds with the attack of the nucleophile to the electrophilic carbon of the isothiocyanate group. Then, a thiourea bond is formed between FITC and the protein without leaving groups.

The present experiment deals with a protein (bovine serum albumin, BSA) modification using a fluorescent probe, i.e. fluorescein isothiocyanate (FITC), and the subsequent determination of the macromolecule modification determined using absorbance spectroscopy. During this experiment students are asked to evaluate the molar extinction coefficient (ε) of the protein (BSA) and the fluorophore (FITC), bioconjugate BSA with FITC and learn how to purify and characterize the obtained conjugate. Students are thus allowed to learn different topics (modification of reactive groups of proteins, preparation, purification, isolation and characterization of conjugates) and use several laboratory techniques (pipetting, gel filtration, spectroscopy).

For its interdisciplinary character, this laboratory experiment is well-suited for students in third year of Bachelor degree in chemistry, biochemistry, biology and biotechnology and the time required for completing the described experiment is no longer than three lab sessions, each one taking about four hours. Students usually work in small groups (ideally two or up to four students each group) depending on the availability of working space and instruments. The accuracy of students' results and response to the experiments were generally good (within 5-10% error).

2. Experimental

2.1. Preparation of Buffer and Reagents

All chemicals were purchased and used without any further purification and distilled water was used for buffer solution preparation.

Phosphate-buffered saline (PBS buffer) solution has to be prepared by students since it is necessary for the preparation of BSA dilutions (1:5, 1:10, 1:20, 1:50, 1:100). For one litre of 10 mM PBS buffer dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 $12H_2O$ and 0.24 g of KH_2PO_4 in 900 ml of distilled water. Then adjust the pH to 7.4 with HCl or NaOH and add water to one liter.

A 0.4 mM stock solution of BSA in PBS buffer is freshly prepared either prior to the lab or by the students at the beginning of the experiment. The BSA stock solution has to be diluted in PBS buffer (1:5, 1:10, 1:20, 1:50, 1:100) by the students.

One stock solution of FITC in DMSO is prepared by the instructors in advance and stored in the refrigerator. The FITC solution is then diluted (1:50, 1.5:100, 1:100, 1:200) with PBS buffer by the students.

2.2. Bioconjugation

The protocol used for the modification of BSA with FITC is reported in literature[1] and is very simple:

1) Students are asked to prepare a 2 mg/ml BSA solution in 0.1 M sodium carbonate, pH 9.
2) FITC is dissolved in dry DMSO at a concentration of 1 mg/ml. (This solution can be prepared by laboratory personnel to avoid contact of students with FITC). Fresh FITC solution has to be used because in old solutions a breakdown of the isothiocyanate group may decrease coupling efficiency. After the preparation, it is important to protect the solution from light by wrapping in aluminium foil or using amber vials.
3) Every student or group took a millilitre of protein solution (2 mg/ml concentration) in a eppendorf. Then, 50-100 µl of FITC solution to each is slowly added and the resulting solution is mixed. The amount of FITC can be varied by every group and can be used to evaluate the D/P variation on the FITC added (see Laboratory Documentation).
4) The solution is let reacting for 8 hours at 4°C in the dark.
5) The reaction is quenched by the addition of ammonium chloride to a final concentration of 50 mM. The eppendorf is left for 2 h. The addition of ammonium chloride is needed to stop the reaction by blocking the remaining unreacted isothiocyanates groups.

The conjugate is then purified by gel filtration (Sephadex G-25) using PBS buffer as eluent.

3. Hazards

Students must wear safety glasses and laboratory coats at all times.

Fluorescein 5-isothiocyanate is harmful and may cause sensitization by inhalation, but FITC stock solution is generally prepared by laboratory personnel in dimethyl sulfoxide (DMSO).

Bovine serum albumin (BSA), BSA-FITC conjugate and the other reagents used to prepare buffer solutions and dilutions are not harmful.

4. Results and Discussion

Students are asked to record the absorbance of the prepared solutions and to calculate the molar extinction coefficient for both BSA and FITC. Then, they perform the bioconjugation experiment, purify the complex by gel filtration and characterize it by means of UV-Vis spectroscopy evaluating the bioconjugation effectiveness.

4.1. BSA Extinction Coefficient Determination

The BSA stock solution (0.40 mM in PBS buffer) is diluted in PBS buffer (1:5, 1:10, 1:20, 1:50, 1:100) by the students and the UV-Vis spectra of all these solutions are recorded (Figure 2).

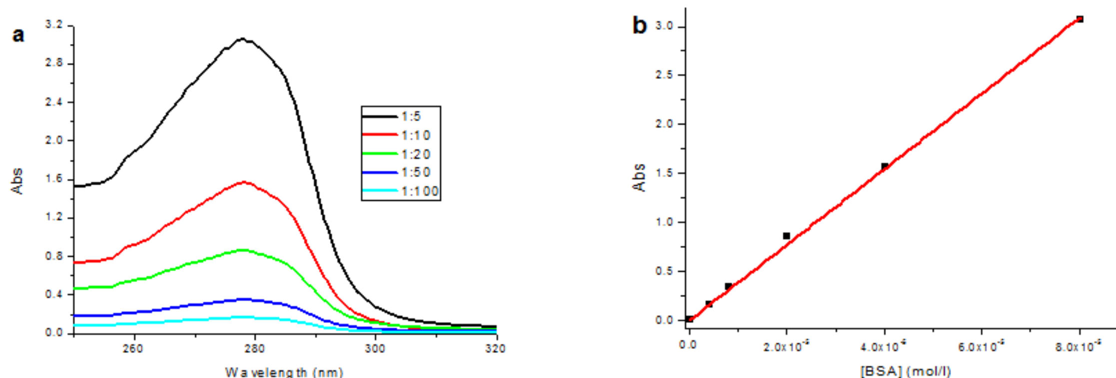

Figure 2. (a) absorbance spectra of the diluted BSA solutions and (b) regression analysis for the determination of the molar extinction coefficient. Data points are the mean of five independent experiments with the standard deviation. Error bars are not visible as they are concealed by the data points

Students are asked to analyse the spectra and to evaluate the values of the maximum of absorbance which are then plotted against the BSA concentration, fitted by a regression line and, from its slope, the molar extinction coefficient is thus calculated applying the Lamber-Beer law.

The obtained $\varepsilon_{protein}$ was 38576 $M^{-1}cm^{-1}$, in agreement with the data reported in literature. [12] The BSA molar extinction coefficient was also determined from knowledge of its amino acid composition and the obtained value was 48150 $M^{-1}cm^{-1}$. [13,14] This is a calculation which is really simple and allows students to experiment with the protein sequence (see Laboratory documentation). Actually, the extinction coefficient of the native protein in water can be computed using equation a: [15]

$$\varepsilon = (\mathbf{nW} \times \mathbf{5500}) + (\mathbf{nY} \times \mathbf{1490}) + (\mathbf{nC} \times \mathbf{125}) \qquad \text{(a)}$$

where n is the number of each tryptophan (W), tyrosine (Y) and cysteine (C) residue respectively and the stated values are the amino acid molar absorptivities at 280 nm.

The difference of the molar extinction coefficient values is not so important if we consider the logε values and that the ε evaluation is solvent and pH dependent.

4.2. FITC Extinction Coefficient Determination

The FITC stock solution (1.0 mM in DMSO) is diluted in PBS buffer (1:50, 1.5:100, 1:100, 1:200) by the students and the UV-Vis spectra of all these solutions are recorded. The obtained values of the maximum of absorbance are plotted against the FITC concentration (see Figure 3) and, from the slope of the regression line, the molar extinction coefficient is calculated. The obtained ε_{dye} was 62348 $M^{-1}cm^{-1}$. This value is in agreement with the molar extinction coefficient reported to be 68000 $M^{-1}cm^{-1}$ in literature at pH 9.2 [16] but FITC is known to be pH sensitive. Actually, it changes spectrum profile and molar extinction coefficient at different pH and solvents [17].

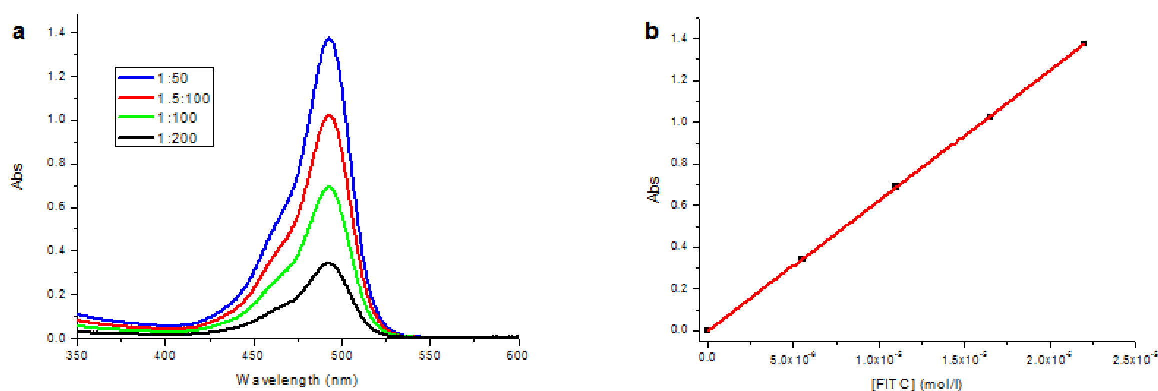

Figure 3. (a) absorbance spectrum of the diluted FITC solutions and (b) regression analysis for the determination of the molar extinction coefficient. Data points are the mean of five independent experiments with the standard deviation. Error bars are not visible as they are concealed by the data points.

4.3. Bioconjugation

The protocol used for the modification of BSA with FITC is reported in literature[1] and in the experimental section. Students take a millilitre of protein solution in a eppendorf. Then, FITC is slowly added and mixed. After 8 hours at 4°C in the dark, the reaction is quenched by the addition of ammonium chloride. The eppendorf is left to react for 2 h to stop the reaction by blocking the remaining isothiocyanates groups.

4.4. Gel Filtration

The conjugate is then purified by gel filtration (Sephadex G-25) using PBS buffer as eluent (Figure 4).

To perform a separation, the first step is the equilibration of the packed bed with buffer. Then, the bioconjugate is eluted isocratically, simply by gravity, and different fractions are collected. The final step consists in a wash using the running buffer in order to remove molecules that might have been held on the column and to prepare the column for a new run. Smaller sized molecules enter the beads in the column while large molecules are excluded and leave the column first, followed by smaller molecules. Sephadex G-25 has a fractionation range for globular proteins of 1000 to 5000 g/mol molecular weights, with an exclusion limit of approximately 5000 g/mol. This means that proteins and peptides larger than 5000 g/mol are therefore easily separated from molecules with molecular weigth of less than 1000 g/mol, which is the case of BSA-FITC and FITC. Free FITC is easily separated from the bioconjugate. Since we work with a considerable FICT excess, there is no free BSA in the reaction mixture.

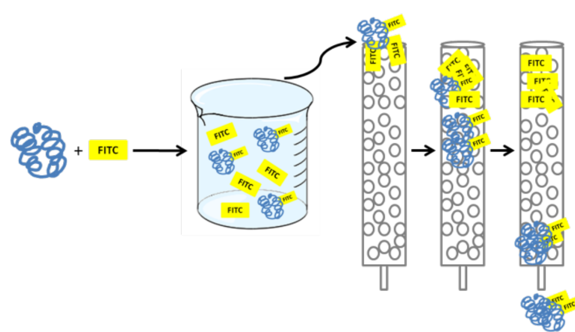

Figure 4. Demonstrative sketch of gel filtration procedure.

4.5. Evaluation of the D/P of the Conjugate

The eluted solutions from the column, after gel separation, are collected in different fractions and analysed by UV-Vis absorption.

The dye/protein ratio (D/P) of the bioconjugate can be determined by the absorption spectra of the labelled proteins, according to the relationship reported in equation 1

[18] and represents the average number of the dye molecules conjugated to each protein molecule:

$$D / P = \frac{A_{\max} \cdot \varepsilon_{prot}}{(A_{280} - cA_{\max}) \cdot \varepsilon_{dye}} \quad (1)$$

where A_{280} is the absorbance of the conjugate at 280 nm; A_{max} is the absorbance of the conjugate at the absorption maximum of the corresponding FITC, c is a correction factor (c = 0.29) which needs to be used in order to adjust for the amount of A_{280} contributed by the dye (this is necessary because fluorescent dyes also absorb at 280 nm) and equals the A_{280} of the dye divided by the A_{max} of the dye; $\varepsilon_{protein}$ and ε_{dye} are the molar extinction coefficients for the protein and the fluorophore used, respectively, which are calculated by the students. A derivation of equation 1 is reported in the Supporting Information.

The dye/protein ratios (D/P) of the conjugates were determined by the absorption spectra of the labelled protein (Figure 5), registered in PBS (pH=7.4) according to equation 1.

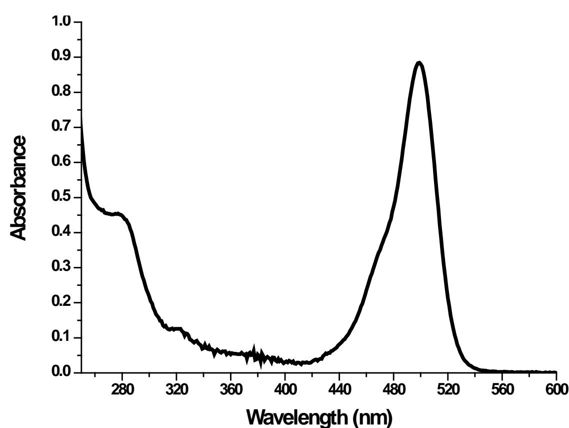

Figure 5. UV-Vis spectrum of the BSA bioconjugated with FITC.

The experiment provided a D/P value around 1.4. The experiment was repeated by several groups of students and the D/P ratio varied from 1.2 to 1.6.

5. Conclusions

Synthesis of bioconjugates involves a variety of challenges, ranging from the simple and nonspecific use of a fluorescent dye marker to the complex design of antibody drug conjugates.

This experiment shows how to bioconjugate a protein (bovine serum albumin, BSA) using a fluorescent probe, i.e. fluorescein isothiocyanate (FITC). A full characterization of the protein, the fluorescent probe and the subsequent conjugate is made by means of UV-Vis spectroscopy. Moreover, students can learn how to purify and characterize the bioconjugate.

This laboratory experience allows students to explore an aspect of bioconjugation chemistry not typically covered in didactical laboratories. Absorption measurements are used to determine the molar extinction coefficient for one protein and one fluorophore and to characterize the bioconjugate. Indeed, this experience also covers unusual aspects of protein bioconjugation, purification and characterization of bioconjugates. The experiment is interdisciplinary, exploring many aspects of biochemistry and can be completed in three four-hour laboratory period.

At the end of the laboratory experience, students are asked to write a report where they have to collect and comment on their data. In this way, along with students' behavior in the lab, the teacher can evaluate the level and the quality of their work and understanding.

Supporting Information

Experimental details, derivation of equation 1, BSA sequence, notes for instructors and students, detailed timetable of the experiment.

References

[1] Hermanson, G. T. Bioconjugate Techniques, 2nd edition, Academic Press, Elsevier, 2008.

[2] Barbero, N.; Barni, E.; Barolo, C.; Quagliotto, P.; Viscardi, G.; Napione, L.; Pavan, S.; Bussolino, F. A study of the interaction between fluorescein sodium salt and bovine serum albumin by steady-state fluorescence. *Dyes Pigments* 2009, *80* (3), 307-313.

[3] Peters T. Serum albumin. *Adv. Prot. Chem.* 1985, *37*, 161-245.

[4] Carter, D. C.; Ho, J. X. Structure of serum albumin. *Adv. Prot. Chem.* 1994, *45*, 153-203.

[5] Barbero, N.; Visentin, S.; Viscardi, G. The different kinetic behaviour of two potential photosensitizers for PDT. *J. Photochem. Photobiol. A: Chem.* 2015, *299*, 38-43.

[6] Williams, K.R.; Adhyaru, B.; Pierce, R.E.; Schulman S.G. The Binding Constant for Complexation of Bilirubin to Bovine Serum Albumin. An Experiment for the Biophysical Chemistry Laboratory. *J. Chem. Educ.* 2002, *79* (1), 115-116.

[7] Liu, J. Investigation of Cu(II) Binding to Bovine Serum Albumin by Potentiometry with an Ion Selective Electrode: A Biophysical Chemistry Experiment for the Undergraduate Curriculum. *J. Chem. Educ.* 2004, *81* (3), 395-397.

[8] Liang, P.; Adhyaru, B.; Pearson, W.L.; Williams K.R. The Binding Constant of Estradiol to Bovine Serum Albumin. An Upper-Level Experiment Utilizing Tritium-Labeled Estradiol and Liquid Scintillation Counting. *J. Chem. Educ.* 2006, *83* (2), 294-295.

[9] Sohl, J. L.; Splittgerber, A. G. The binding of Coomassie brilliant blue to Bovine Serum Albumin: A physical biochemistry experiment. *J. Chem. Educ.*, 1991, *68* (3), 262-264.

[10] Podhradský, D.; Drobnica, L.; Kristian, P. Reactions of cysteine, its derivatives, glutathione coenzyme A, and dihydrolipoic acid with isothiocyanates. *Experientia* 1979, *35 (2)*, 154-155.

[11] Jobbágy, A.; Király, K. Chemical characterization of fluorescein isothiocyanate-protein conjugates. *Biochym. Biophys. Acta* 1966, *124* (1), 166-175.

[12] https://tools.lifetechnologies.com/content/sfs/brochures/TR0006-Extinction-coefficients.pdf Extinction Coefficients, last accessed date: 2016, May 25th.

[13] Gill, S. C.; von Hippel, P.H. Calculation of Protein Extinction Coeffients from Amino Acid Sequence Data. *Anal. Biochem.* 1989, *182 (2)*, 319-326.

[14] http://www.basic.northwestern.edu/biotools/proteincalc.html Peptide Property Calculator, last accessed date: 2016, May 25th.

[15] Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.* 1995, *4* (11), 2411-2423.

[16] Ambrosini, S.; Beyazit, S.; Haupt, K.; Tse Sum Bui, B. Solid-phase synthesis of molecularly imprinted nanoparticles for protein recognition. *Chem. Commun.* 2013, *49 (60)*, 6746-6748.

[17] Hadjmohammadi, M. R.; Chaichi, M. J.; Yousefpour, M. Solvatochromism Effect of Different Solvents on UV-Vis Spectra of Flourescein and its Derivatives. *Iran. J. Chem. Chem. Eng.* 2008, *27* (4), 9-14.

[18] Barbero, N.; Napione, L.; Quagliotto, P.; Pavan, S.; Barolo, C.; Barni, E.; Bussolino, F.; Viscardi, G. Fluorescence anisotropy analysis of protein–antibody interaction. *Dyes Pigments* 2009, 83 (2), 225-229.

SUPPORTING INFORMATION

Derivation of equation 1 [1]

$$D/P = \frac{A_{\max} \cdot \varepsilon_{prot}}{(A_{280} - cA_{\max}) \cdot \varepsilon_{dye}} \quad (1)$$

The D/P ratio can be calculated by using equation 2:

$$D/P = \frac{A_{\max}}{\varepsilon_{dye} \cdot [P]} \quad (2)$$

where [P] is the protein molar concentration which can be derived by equation 3:

$$[P] = \frac{A_{280} - (c \cdot A_{\max})}{\varepsilon_{prot}} \quad (3)$$

If we substitute [P] equation 2 becomes equation 1a:

$$D/P = \frac{A_{\max}}{\varepsilon_{dye} \cdot \left(\frac{A_{280} - (c \cdot A_{\max})}{\varepsilon_{prot}} \right)} \quad (1a)$$

$$= \frac{A_{\max} \cdot \varepsilon_{prot}}{\varepsilon_{dye} \cdot \left(A_{280} - c \cdot A_{\max} \right)}$$

BSA sequence:

MKWVTFISLLLLFSSAYSRGVFRRDTHKSEIAHRF
KDLGEEHFKGLVLIAFSQYLQQCPFDEHVKLVNELT
EFAKTCVADESHAGCEKSLHTLFGDELCKVASLRE
TYGDMADCCEKQEPERNECFLSHKDDSPDLPKLKP
DPNTLCDEFKADEKKFWGKYLYEIARRHPYFYAPE
LLYYANKYNGVFQECCQAEDKGACLLPKIETMREK
VLASSARQRLRCASIQKFGERALKAWSVARLSQKF
PKAEFVEVTKLVTDLTKVHKECCHGDLLECADDRA
DLAKYICDNQDTISSKLKECCDKPLLEKSHCIAEVE
KDAIPENLPPLTADFAEDKDVCKNYQEAKDAFLGS
FLYEYSRRHPEYAVSVLLRLAKEYEATLEECCAKD
DPHACYSTVFDKLKHLVDEPQNLIKQNCDQFEKLG
EYGFQNALIVRYTRKVPQVSTPTLVEVSRSLGKVGT
RCCTKPESERMPCTEDYLSLILNRLCVLHEKTPVSE
KVTKCCTESLVNRRPCFSALTPDETYVPKAFDEKLF
TFHADICTLPDTEKQIKKQTALVELLKHKPKATEEQ
LKTVMENFVAFVDKCCAADDKEACFAVEGPKLVV
STQTALA

BSA Average Mass: 66432.96 g/mol

Gel filtration

Size-exclusion chromatography (SEC) is a chromatographic method that separates molecules according to differences in size, and in some cases molecular weight, as they pass through a SEC medium packed in a column. [2] Unlike other kinds of chromatographic techniques, molecules do not bind to the chromatography medium. In this way, buffer composition does not directly affect the degree of separation. SEC is usually applied to large molecules or macromolecular complexes such as proteins and polymers. The main application of SEC concerns the removal of small molecules from a group of larger molecules. Small molecules such as excess salt or free labels are easily separated from larger molecules. In this case, gel filtration is needed because fluorescent dyes can bind noncovalently to proteins. The use of gel filtration will remove any nonspecifically bound dye.

A porous matrix of spherical particles with chemical and physical stability and inertness is packed into a column to form a packed bed. For greater convenience, we used a prepacked Sephadec G-25 column. To perform a separation, the first step is the equilibration of the packed bed with buffer. Then, the bioconjugate is eluted isocratically, simply by gravity, and different fractions are collected. The final step consists in a wash using the running buffer in order to remove molecules that might have been held on the column and to prepare the column for a new run. Smaller sized molecules enter the beads in the column while large molecules are excluded and leave the column first, followed by smaller molecules. Sephadex G-25 has a fractionation range for globular proteins of 1000 to 5000 g/mol molecular weights, with an exclusion limit of approximately 5000 g/mol. This means that proteins and peptides larger than 5000 g/mol are therefore easily separated from molecules with molecular weigth of less than 1000 g/mol, which is the case of BSA-FITC and FITC. Free FITC is easily separated from the bioconjugate. Since we work with a considerable FICT excess, there is no free BSA in the reaction mixture.

Absorption Measurements

UV-Vis measurements were recorded using a Cary 300 Spectrophotometer with a working range past 5.0 absorbance units (Abs).

Hazards

Fluorescein 5-isothiocyanate is harmful and may cause sensitization by inhalation but FITC stock solution is generally prepared by laboratory personnel. Solutions can be prepared in *N*,*N*-dimethylformamide (DMF) or dimethyl sulfoxide (DMSO). DMSO is preferred because DMF has been shown to have carcinogenic and mutagenic properties.

Bovine serum albumin (BSA), BSA-FITC conjugate and the other reagents used to prepare buffer solutions and dilutions are not harmful.

In any case, personal protective equipment (lab coat, gloves and goggles) must be worn in the lab.

Instructors' Notes:

The instructors have to prepare a stock solution of FITC in DMSO (1 mg/ml) in advance that should be stored in the refrigerator until the experiment. This is to avoid any contact of the students with FITC powder. The FITC solution is then diluted (1:50, 1.5:100, 1:100, 1:200) with PBS buffer by the students. Fresh FITC solution has to be used because breakdown of the isothiocyanate group over time may decrease coupling efficiency. After the preparation of the solution, it is important to protect it from light by wrapping in aluminium foil or using amber vials. Do not store it for more than three days.

A 0.4 mM stock solution of BSA in PBS buffer has to be prepared fresh either prior to the lab or by the students at the start of the experiment. Be careful to use the solution after letting it to rest for 1-2 hours in order to avoid the presence of bubbles formed by BSA dissolution.

The amount of FITC for the bioncojugation experiment can be varied by every group and can be used to evaluate the D/P variation on the FITC added. We usually prepare a table with the D/P values and the BSA/FICT ratio. This value normally does not change by varying the BSA/FITC ratio.

The bioconjugation needs around 8 hours to react. Students usually prepare the reaction mixture in the morning, right after their arrival in the lab, and they stop the reaction at the end of the lab, after 8 hours. Otherwise, if the lab is only in the afternoon, they start the reaction and they leave it overnight. We saw no dependence on the reaction time on the D/P ratio in this range of time.

The same experiment can be performed using human serum albumin (HSA) instead of BSA. HSA is now available at low cost. Some groups can work with BSA, some other with HSA. Students can also discuss the difference between BSA and HSA bioconjugation.

UV-Vis measurements were recorded using a Cary 300 Spectrophotometer with a working range past 5.0 absorbance units (Abs). If another instrument with different working range is used, the concentrations used need to be changed in order to be in the correct Abs range.

Students' Handbook

Students are asked to prepare a phosphate-buffered saline (PBS buffer) solution which is used for all the dilutions of the experiment. For one litre of 10 mM PBS buffer dissolve 8 g of NaCl, 0.2 g of KCl, 1.44 g of $Na_2HPO_4 \cdot 12H_2O$ and 0.24 g of KH_2PO_4 in 900 ml of distilled water. Then adjust the pH to 7.4 with HCl or NaOH and add water to one liter.

Students have to prepare the following BSA dilutions in PBS: 1:5, 1:10, 1:20, 1:50, 1:100 as well as FITC dilutions: 1:50, 1.5:100, 1:100, 1:200 with PBS buffer.

The BSA stock solution, provided by the instructors, has to be diluted in PBS buffer (1:5, 1:10, 1:20, 1:50, 1:100) by the students and the UV-Vis spectra of all these solutions have to be recorded. Students are asked to analyse the spectra and to evaluate the values of the maximum of absorbance which are then plotted against the BSA concentration, fitted by a regression line and, from its slope, the molar extinction coefficient is thus calculated applying the Lamber-Beer law.

The FITC stock solution (1.0 mM in DMSO), provided by the instructors, has to be diluted in PBS buffer (1:50, 1.5:100, 1:100, 1:200) by the students and the UV-Vis spectra of all these solutions are recorded. The obtained values of the maximum of absorbance have to be plotted against the FITC concentration and, from the slope of the regression line, the molar extinction coefficient is calculated.

For the bioconjugation experiments, see the details in the experimental section.

Detailed Timetable

We performed this experience in three lab sessions (each one of 4 h).

1. *Evaluation of the BSA molar extinction coefficient*: preparation of the solutions, absorbance measurements, analysis of the spectra and evaluation of the values of the maximum of absorbance which are then plotted against the BSA concentration, fitted by a regression line and, from its slope, the molar extinction coefficient is thus calculated applying the Lamber-Beer law. Evaluation of the BSA molar extinction coefficient from knowledge of its amino acid composition.
2. *Evaluation of the FITC molar extinction coefficient*: preparation of the solutions, absorbance measurements, analysis of the spectra and evaluation of the values of the maximum of absorbance which are then plotted against the FITC concentration, fitted by a regression line and, from its slope, the molar extinction coefficient is thus calculated applying the Lamber-Beer law.
3. *Biocojugation* and characterization of the BSA-FITC complex*: purification of the bioconjugate by gel filtration (Sephadex G-25). The eluted solutions after purification are fractionated and analysed by UV-Vis absorption. Determination of the dye/protein ratio of the conjugate.

* Bioncojugation needs 8 hours and has to be launched during the first two lab sessions and let react in the fridge.

References

[1] https://tools.thermofisher.com/content/sfs/brochures/TR0031-Calc-FP-ratios.pdf Calculate dye:protein (F/P) molar ratios, last accessed date: 2016, June 3rd

[2] Hong, P., Koza, S., Bouvier, E. S. P. A Review. Size-Exclusion Chromatography For The Analysis Of Protein Biotherapeutics And Their Aggregates. *J. Liq. Chrom. Relat. Tech.* 2012, *35*, 2923-2950.