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Conjugation of spin labeled proteins to fluorescent quantum dots*

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Abstract

Biocompatible nanomaterials functionalised with proteins have a high potential for diagnostics and therapy. Fluorescent quantum dots covalently coupled to functional proteins are of particular interest. The covalent binding to the functionalised ligands of the quantum dot polymer coating is a critical step because it has to be most efficient without affecting the protein conformation and function. In the present study we report on the application of EPR spectroscopy in the investigation of the bioconjugation process of fluorescent quantum dots. Our particular interest is the quantification of potential binding sites on the nanoparticle coating. After spin labeling, we determined 80+/-7 binding sites on an amino-functionalised quantum dot. Moreover, EPR spectroscopy allowed to quantify the amount of spin labeled recombinant human annexin V coupled to a carboxyl-functionalised quantum dot.

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Keywords: Quantum dots; bioconjugation; annexin V; electron paramagnetic resonance (EPR)

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Nomenclature

APS	ammonium peroxodisulfate
DDT	dithiothreitol
EDC	1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride
EDTA	disodium ethylenediaminetetraacetate
EPR	electron paramagnetic resonance
IAASL	3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy
PEG	polyethylene glycol
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sulfo-GMBS	N-γ-maleimidobutyryl-oxysulfosuccinimide ester
TAE	Tris-acetate-EDTA
TEMED	N, N, N´, N´-tetramethylenediamide
TEMPO	2,2,6,6-tetramethylpiperidine 1-oxyl
2-IT	Traut´s reagent, 2-iminothiolane

1. Introduction

Biocompatible nanomaterials functionalised with proteins have a high potential for diagnostics and therapy. Fluorescent quantum dots covalently coupled to functional proteins are of special interest because they enable tracing of physiological dynamics in living cells with high spatial resolution. The covalent coupling of proteins to the functionalised coating of water soluble quantum dots is a critical step because it may interfere with the integrity of the protein conformation and function (1). Thus, the evaluation of the protein conformation and conformational dynamics upon binding to the nanoparticle surface is essential for quality control. In addition, such investigations will foster our understanding of the interaction of nanostructured or functionalised surfaces with proteins. Recent developments including pulse techniques and new spin labeling techniques have made electron paramagnetic resonance (EPR) spectroscopy a powerful approach for studying the conformation and conformational dynamics of proteins in biomolecular complexes and biomolecule-nanomaterial hybrid systems (2, 3).

In the present study, we report on the application of EPR spectroscopy in bioconjugation of quantum dots with a special focus on the quantification of potential binding sites on the nanoparticles as well as on the quantification of protein molecules bound per quantum dot upon bioconjugation. Using Traut`s reagent (2-iminothiolane, 2-IT), water soluble nanoparticles were spin labeled with the 3-(2-Iodoacetamido)-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (IAASL) radical. EPR spectroscopy revealed the number of binding sites on the functionalized surface coating of the quantum dots. Recombinant human annexin V was chosen as model protein for bioconjugation and spin labeled with IAASL. After conjugation to the quantum dots with 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), gel electrophoresis and EPR spectroscopy of the quantum dot–annexin V constructs showed successful bioconjugation. A double integration of the EPR spectrum allowed for the quantification of annexin V molecules bound per quantum dot.

2. Materials and methods

2.1. Materials

All chemicals were purchased from Applichem or Carl Roth GmbH & Co. KG and not purified further if not specifically mentioned otherwise. Human recombinant annexin V was obtained from eBioscience (San Diego, CA, USA). Qdot® 655 ITK™ carboxyl and Qdot® 655 ITK™ amino (PEG) quantum dots, 1-ethyl-3-(3-Dimethylaminopropyl)carbodiimide (EDC), Trauts' Reagent (2-imino-thiolane, 2-IT) and N-γ-maleimidobutyryl-oxysulfosuccinimide ester (sulfo-GMBS) were purchased from ThermoFisher scientific.

The following buffers are common to the protocols presented in this work:

Buffer A: 500 mM sodium phosphate, 750 mM NaCl, 25mM EDTA, pH 7.2.
Buffer B: 100 mM sodium phosphate, 150 mM NaCl, 5 mM EDTA, pH 8.
Buffer C: same as buffer B but containing 6 M urea.
Buffer D: 50 mM borate, pH 8.3.
Buffer E: 10 mM borate, pH 7.4.
Stock solution of annexin V (162 µM) in buffer B.

All buffers were freshly prepared and filter sterilised through a 0.2 micron filter (Merck Millipore) before use.

2.2. Functionalisation of quantum dots with sulfhydryl groups and spin labels

Qdot® 655 ITK™ amino (PEG) quantum dots were functionalised with 2-IT to modify the primary amines with sulfhydryl (-SH) groups. For this purpose, 8 µL of an 8 µM quantum dot solution was diluted with 30 µL of buffer A and 30 µL of buffer B. Traut's Reagent was added in a quantum dot to 2-IT ratio of 1 to 800. The sample was incubated for 1 h at room temperature and washed from unbound 2-IT with buffer B using Zeba spin desalting columns (ThermoFisher scientific). The sulfhydryl-modified quantum dots were incubated with 1mM DDT for 1 h, washed with buffer B using desalting columns, reacted with IAASL for 20 h at 4°C and washed again with buffer B. The spin labeled quantum dots were stored in buffer D.

2.3. Spin labeling of annexin V

Human recombinant annexin V (eBioscience, San Diego, CA, USA) was unfolded by dilution to a 20 µM solution in buffer C and incubated with 10 mM dithiothreitol (DDT) for 1 h. The solution was purified from DDT by washing with buffer C using centrifugal filter units (Amicon filters) with 10 kDa molecular weight cutoff (Amicon/Millipore, Carringtwohill, Co. Cork, Ireland). For spin labeling the protein was incubated with 1 mM IAASL for 20 h at 4°C. The sample was then purified from unbound spin labels and annexin V was refolded by washing with buffer B using 10 kDa Amicon filters.

2.4. Conjugation of quantum dots with spin labeled annexin V

Before the conjugation reaction, the quantum dots and the protein were dialyzed each for 1 h against buffer E using Slide-A-Lyzer® dialysis cassettes with a molecular weight cut off of 3.5 kDa. Qdot® 655 ITK™ carboxyl quantum dots and spin labeled annexin V were incubated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) to couple the primary amines of annexin V to the quantum dot carboxyl group. The reaction was carried out in buffer E with a total volume of 300 µl. 160 pmol of quantum dots and 9.6 nmol of spin labeled annexin V (1:60 ratio) were applied. EDC was dissolved in buffer E and quickly added to the reaction mixture to a final concentration of 0.8 mM. The reaction mixture was protected from light and stirred for 2 h at room temperature. The product was purified by three washing steps with buffer D using a Vivaspin® centrifugation unit with a molecular weight cut off of 50 kDa (GE Healthcare Life Sciences). The final concentration of quantum dots in the product was determined by UV/VIS spectrometry at wavelength λ = 405 nm with a molar extinction coefficient ε = 9.900.000 $cm^{-1}M^{-1}$ as specified by the quantum dot manufacturer. The product yield was 87% of the quantum dot amount applied to the reaction.

2.5. Agarose gel electrophoresis for protein-quantum dot conjugate analysis

For the analysis of the protein-quantum dot conjugate, a gel was produced by melting 1.95 g agarose (GTQ Roti®Garose for DNA/RNA electrophoresis, Carl Roth GmbH&Co. KG, Karlsruhe, Germany) in 130 ml Tris-acetate-EDTA (TAE) buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.5). After cooling to 60°C, 1.3 ml

of a 10% (w/v) SDS solution were added to the agarose solution. The gel was cast in a 15 cm x 13.9 cm casting tray (MSCHOICE, Kisker Biotech GmbH&Co. KG, Steinfurt, Germany). A comb was inserted to mould the sample wells. The samples were prepared as follows: 5.6 µmol of quantum dot or quantum dot conjugate respectively in a total volume of 30 µl buffer D were mixed with 10 µl of sample buffer (30% (v/v) TAE buffer, 1% (w/v) SDS, 50% (v/v) glycerol, 0.01% (w/v) bromphenol blue). A volume of 25 µl of each sample was loaded onto the gel. The gel was run in TAE buffer supplied with 1% (w/v) SDS at 80 V for 70 min. After the run, the gel was placed on a UV-transilluminator (BIO View UV light UXFT-20MW-8R, biostep® GmbH, Germany) and documented with an INTAS Gel-Jet-Imager Kit Type 2000 (Intas GmbH, Göttingen, Germany).

2.6. SDS-PAGE and coomassie staining

To analyse the bioconjugate for complete removal of unbound protein, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed. In brief, a two-phase gel consisting of a 4% acrylamide stacking gel (0.5 ml acrylamide/bisacrylamide solution (37.5:1), 0.75 ml stacking gel buffer (0.5 M Tris, 0.4% (w/v) SDS, pH 6.8), 1.75 ml ddH_2O, 12 µl N, N, N´, N´-tetramethylenediamide (TEMED), 12 µl 10% (w/v) Ammonium peroxodisulfate solution (APS)) and a 10% acrylamide separating gel (2.0 ml acrylamide/bisacrylamide solution (37.5:1), 1.5 ml seperating gel buffer (1.5 M Tris, 0.4%(w/v) SDS, pH 8.8), 1.5 ml ddH_2O, 12 µl TEMED, 12 µl 10 % (w/v) APS) was cast between two glass plates in a gel caster (Biometra GmbH, Göttingen, Germany) and polymerized. Defined amounts of sample or annexin V standards respectively were prepared with 4x non-reducing loading buffer (125 mM Tris, 3% (w/v) SDS, 50% Glycerin, 0.01% bromphenol blue, pH 6.8) and run on the SDS-PAGE at a current of 15 mA for 1.5 h. To determine the size of the proteins, a protein ladder was also applied (color prestained protein standard, broad range, 11-245 kDa, New England Biolabs, Ipswich, MA, USA).

The lanes were loaded as follows:

lane 1: 0 µg human annexin V standard
lane 2: 1 µg human annexin V standard
lane 3: 2 µg human annexin V standard
lane 4: 3 µg human annexin V standard
lane 5: an amount of the conjugate equal to 3.86 µg spin labeled human annexin V as determined by EPR spectroscopy
lane 6: prestained protein marker

For the coomassie staining, the stacking gel was removed. The separating gel was stained for 30 min in staining solution (0.275 % (w/v) Coomassie Brilliant Blue R250, 10% acetic acid, 50% ethanol) and destained in destainining solution (7% acetic acid, 10% ethanol) until clear blue bands were visible. The gel was placed on a VIS-transilluminator (BIO View UV light UXFT-20MW-8R, biostep® GmbH, Germany) and documented with an INTAS Gel-Jet-Imager Kit Type 2000 (Intas GmbH, Göttingen, Germany).

2.7. EPR spectroscopy

X-band cw EPR experiments were performed on a custom built EPR spectrometer equipped with a Bruker dielectric resonator. The microwave power was set to 1.0 mW, the B-field modulation amplitude was 0.15 mT. Glass capillaries of 0.9 mm inner diameter were filled with a sample volume of 15 µl, sufficient to fill the total active resonator volume of 10 µl. Spin concentrations were determined by double integration of the EPR spectrum and comparison with a standard spin label (100 µM 2,2,6,6-tetramethylpiperidine 1-oxyl, TEMPO).

3. Results and discussion

3.1. Quantification of binding sites on a sulfhydryl-modified quantum dot surface

The number of SH-modified amines on a quantum dot surface (Qdot® 655 ITK amino (PEG)) is determined here by measurement of the spin label (IAASL) concentration of spin labels reacted with the SH groups using EPR spectroscopy. IAASL has been frequently used for site-directed spin labeling of proteins, where the reaction of SH groups of engineered cysteines with IAASL was shown to yield quantitative spin labeling (4,5). Quantum dots were first functionalised with Traut's Reagent to modify the primary amines with sulfhydryl (-SH) groups and IAASL was then reacted with these SH groups (Fig.1).

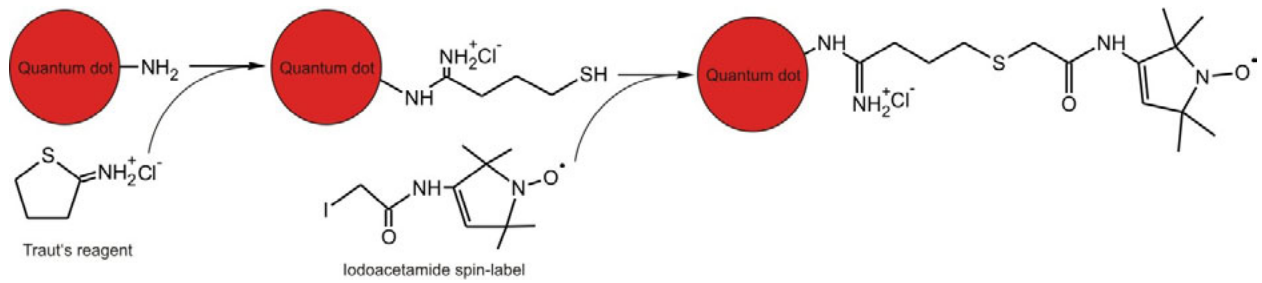

Fig. 1. Reaction scheme of sulfhydryl-modification by Traut´s reagent and spin labeling with the iodoacetamide spin label IAASL.

The EPR spectrum of the spin labeled quantum dots reveals three sharp lines of similar amplitude (Fig. 2) indicating high reorientational freedom of the bound nitroxides (6,7,8) due to the long flexible linkers they are bound to.

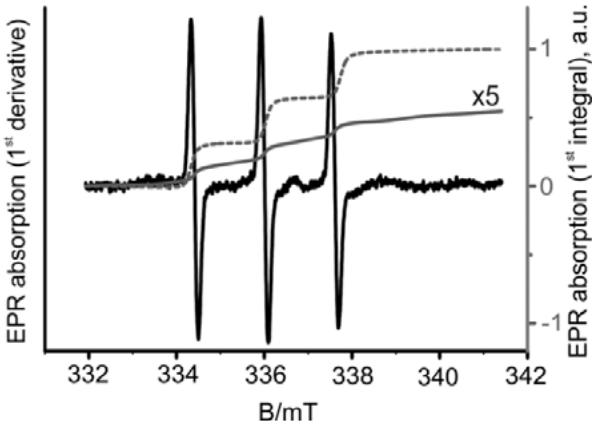

Fig. 2. EPR spectrum of spin labeled quantum dots (1st derivative (black) and 1st integral (continuous gray line) of the EPR absorption). The integral of the EPR absorption is a measure of the spin concentration and compared to that of a 100 µM TEMPO solution (dotted gray) recorded under the same conditions. The shown sample with a quantum dot concentration of 0.14 µM yields a spin concentration of 11 µM.

The area under an EPR absorption spectrum is proportional to the number of spins contributing. Thus, the ratio of the double integrals of the recorded 1st derivative EPR spectra of the sample and of a standard (100 µM TEMPO), multiplied by the concentration of the standard, yields the spin concentration of the sample. Experiments with quantum dots in the concentration range between 0.07 and 0.14 µM yielded an average number of 80+/-7 spin labels bound per quantum dot. This was in good agreement with the specification of the manufacturer who proposed 80-100 binding sites per nanoparticle.

3.2. Conjugation of quantum dots with annexin V

Spin labeling of annexin V in its native state resulted in labeling efficiencies of less than 10%. As illustrated in the ribbon model of the protein, the only native cysteine at position 316 is buried in the protein interior and thus it is poorly accessible for the spin label (Fig. 4a).

For further experiments the protein was spin labeled in its unfolded state induced by 6 M urea (9). Upon removal of urea the clear protein solution revealed proper refolding. The EPR spectrum (not shown) revealed a nitroxide spectrum in the intermediate motional regime characteristic for a spin label undergoing tertiary interaction as expected for a buried spin label binding site (7,8). Double integration of the EPR spectrum yielded a labeling efficiency up to 78%.

The spin labeled annexin V was then bound to Qdot® 655 ITK™ carboxyl quantum dots by incubation of the quantum dot–annexin V mixture with EDC. In this reaction, the carboxyl group of the functionalised quantum dot forms a reactive o-acylisourea intermediate with EDC, which then reacts with primary amino groups of the annexin V molecule, resulting in a covalent amide bond (Fig 3).

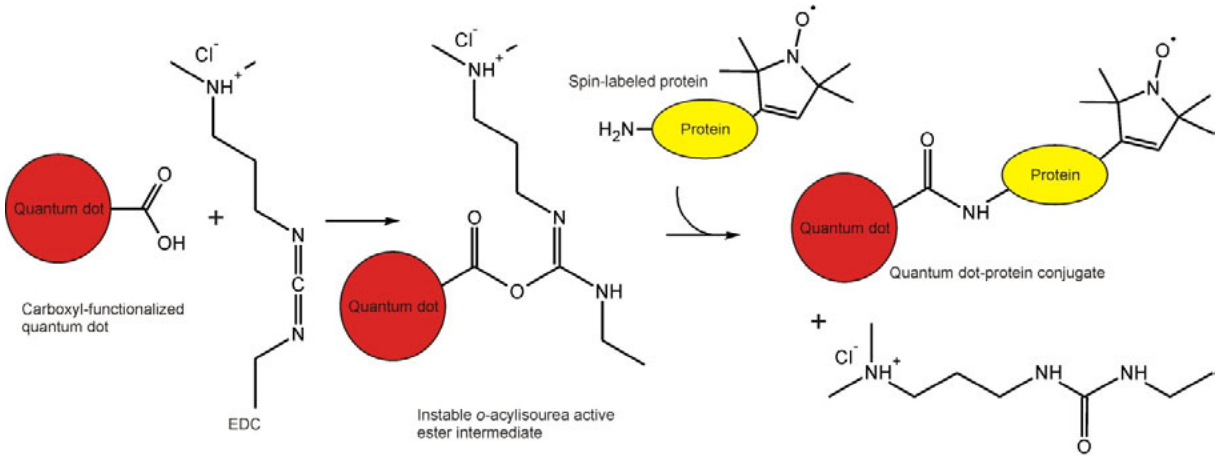

Fig. 3. Reaction scheme of the EDC-mediated conjugation of carboxyl functionalised quantum dots with the amino groups of annexin V.

The success of the bioconjugation was analysed by agarose gel electrophoresis. Compared to the unconjugated quantum dot, the conjugate showed a shift in the electrophoretic mobility to a lower migration rate, which clearly indicates protein binding to the nanoparticle (Fig 4b).

The EPR spectrum of annexin V after conjugation to the quantum dot showed a nitroxide spectrum in the intermediate motional regime with increased mobility as compared to the free spin labeled annexin V (Fig. 4c). However, SDS-PAGE showed that the mixture was not completely purified from unbound protein (Fig. 4d). Therefore, it remained unclear if only the fraction of conjugated annexin V had undergone a conformational change, or if the unbound annexin V was also changed to some extent. While the first finding would suggest a conformational change upon close contact with the quantum dot, the second finding might be led back to intramolecular conjugation of amino acid residues of the annexin V protein by EDC. The conformational change of annexin V in the conjugation to water soluble quantum dots will be the object of future investigations.

Double integration of the EPR spectrum yielded in an average binding number of apparently 31 annexin V molecules per quantum dot. While SDS-PAGE showed that the mixture was not free from unbound protein, the amount of free annexin V present in the conjugate solution was estimated between 1-2 µg from the standards (Fig. 4d, lane 2, 3 compared to lane 5), resulting in an amount of 25-50% unbound protein. Therefore, the average number of bound annexin V molecules per nanoparticle was determined as 19+/-4.

In order to verify this number, we calculated the number of theoretical binding sites based on a simple sterical model with quantum dot and annexin V as spherical particles. The supplier provided the information that the

core/shell/polymer coating complex of the Qdot® 655 ITK™ carboxyl nanoparticle has an average radius of 9 nm. Using the software Dynamics^{TM} V7.1.8.93 of Wyatt Technology Corporation, a radius r_{Anx} = 2.7 nm was estimated for annexin V. In a spherical model, the median sphere of quantum dot covered with annexin V therefore has the radius $r = r_{Qdot} + r_{Anx}$= 11.7 nm resulting in a surface area $A = 4\pi r^2$ = 1719.3 nm^2. If this area is covered by annexin V in a squarish pattern, the area occupied a equals $(2r_{Anx})^2$ = 29.2 nm^2 per annexin V. This would yield A/a=58 sterically accessible sites. Compared to 19 ± 4 bound protein molecules per nanoparticle found experimentally this yields 30 % binding efficiency in the coupling reaction. However, likely deviations from the assumed ideal spherical shapes and the squarish packing pattern change the theoretical number of sterically allowed binding sites.
Furthermore, the simple model does not take into account that the functional carboxyl groups might not be evenly distributed on the quantum dot surface and that a considerable fraction of B = 80 binding sites are sterically shielded by bound proteins, because the average distance between the binding sites $d = \sqrt{(A/B)}$ = 4.6 nm is less than $2r_{Anx}$,= 5.4 nm. Thus, a coverage of 30% of the nanoparticle surface by proteins appears to be reasonable.

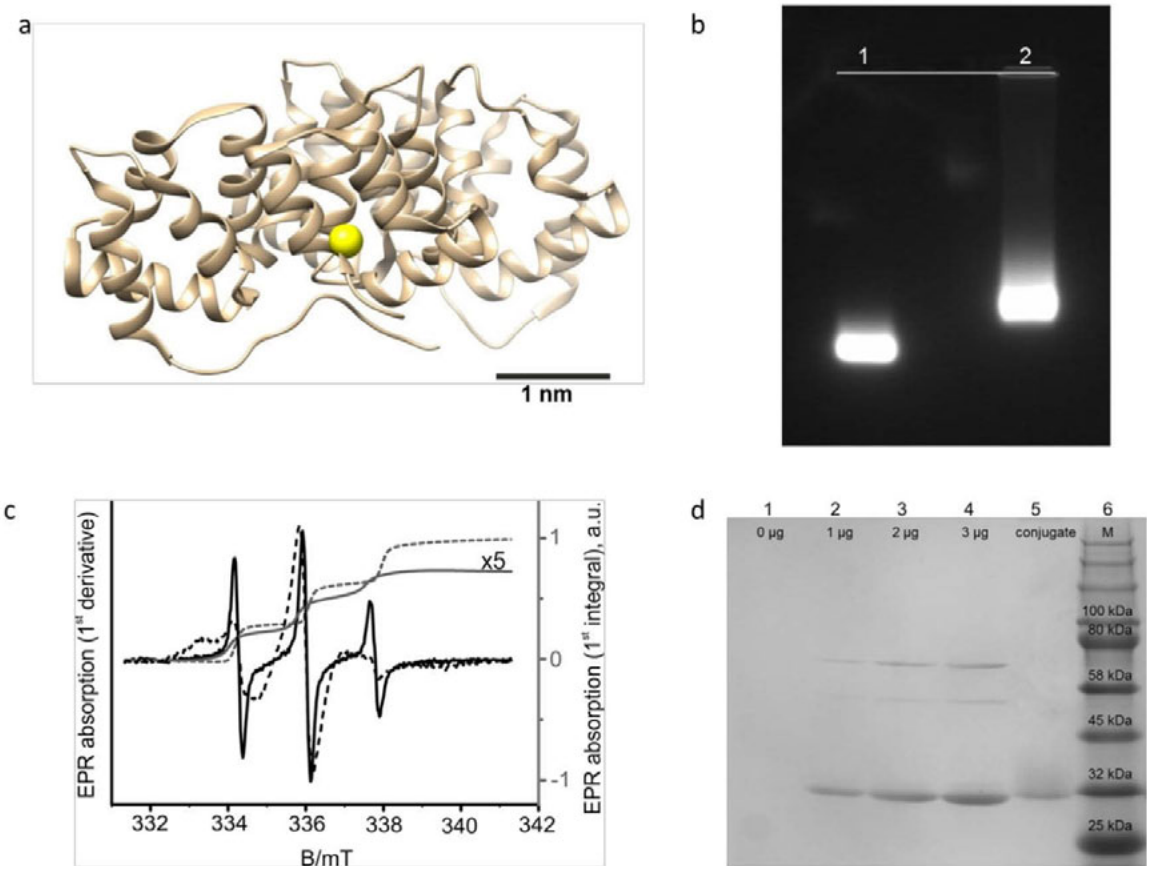

Fig. 4. (a) Ribbon model of the annexin V molecule. At position 316 the IAASL is bound to the native cysteine, which is marked by a yellow sphere. The graphic was designed with UCSF Chimera (www.cgl.ucsf.edu/chimera/); (b) Agarose gel of the unconjugated quantum dot (lane 1) and the conjugate (lane 2); (c) EPR spectrum of the spin labeled annexin V before (dashed black) and after (continuous black) conjugation to the quantum dots: 1st derivative (black) and 1st integral of the EPR absorption (continuous gray line) compared to that of a 100 µM TEMPO solution (dotted gray); (d) SDS-PAGE of the conjugate showing an estimated amount of 1-2 µg unbound protein in the conjugate.

4. Conclusion

EPR spectroscopy is a powerful technique with high potential to support the development and optimisation of bioconjugation protocols especially to fluorescent nanoparticles. In the present study, the application of EPR led to the determination of 80+/-7 binding sites on an amino-functionalised quantum dot. In a second approach, we validated the success of a bioconjugation procedure between the spin labeled model protein annexin V and a carboxyl functionalised quantum dot with EPR. We were able to quantify the average molecule number of spin labeled annexin V bound per quantum dot as 19+/-4. Moreover, the comparison of the EPR spectra of spin labeled annexin V before and after the conjugation revealed a conformational change that will be further investigated in the future.

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