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In: *Trac-trends in Analytical Chemistry* 83: 31–48, 2016.

To refer to or to cite this work, please use the citation to the published version:

Foubert, Astrid, Natalia Beloglazova, Andreja Rajkovic, Benedikt Sas, Annemieke Madder, Irina Yu Goryacheva, and Sarah De Saeger. 2016. "Bioconjugation of Quantum Dots : Review & Impact on Future Application." *Trac-trends in Analytical Chemistry* 83: 31–48. https://doi.org/10.1016/j.trac.2016.07.008

Bioconjugation of quantum dots: Review & impact on future application

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Abstract

Nowadays luminescent semiconductor quantum dots (QDs) are widely applied in different areas due to their unique optical properties. QDs can be used as photoluminescent labels with excellent possibilities for high-throughput detection and diagnostics. For most of such applications QDs must be coupled to biomolecules, which often represents a fundamental challenge. Although QDs have a lot of advantages over organic dyes, most of the techniques that have been developed for QD functionalization and bioconjugation, are more complicated than the corresponding techniques for organic fluorescent dyes. Here, the importance of choosing a suitable bioconjugation strategy in different applications, such as imaging and assays is described. The main goal of this review is to give a structured and detailed overview and comparison of the most widely used conjugation strategies in function of the active groups (carboxyl, amine, thiol, epoxy, hydroxyl and aldehyde groups) present on QD surface.
Keywords: quantum dots, bioconjugation, biolabeling, immobilization of biomolecules, conjugation strategies, non-covalent binding, dative binding, covalent binding.

Abbreviations: ADH, adipic acid dihydrazide; bp, base pair; BSA, bovine serum albumin; CDI, N, N'-carbonyldiimidazole; CNBr, cyanogen bromide; dBSA, denatured BSA; DCC, N,N'-dicyclohexyl carbodiimide; DSP, dithiobis(succinimidylpropionate); DTT, dithiothreitol; DHLA, dihydrolipoic acid; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; EG7: hepta(ethyleneglycol) succinimidylpropionate disulfide; ELISA, enzyme linked-immunosorbent assays; FRET, fluorescence resonance energy transfer; GSH, glutathione; His, histidine; Ig, immunoglobulin; K_D, dissociation constant; LFIA, lateral flow immunoassays; LC-SPDP, long chain SPDP; MAA, mercaptoacetic acid; 2-MAE, 2-mercaptoethylamine; MBP, maltose-binding protein; MPA, with 3-mercaptopropionic acid; MPA-NHS, 3-maleimidopropionic acid NHS; MPS, mercaptopropyltris(methoxy)silane; Ni-NTA, nickelnitrilotriacetic acid; PEG, polyethylene glycol; pI, isoelectric point; PL, photoluminescence; PMAO, poly(maleic anhydride 1-octadecene); PMPI, p-maleimidophenyl isocyanate; QD, quantum dot; QD-COOH, carboxyl functionalized QDs; QD-NH_2, amine functionalized QD; QD-OH, hydroxyl functionalized QD; QD-SH, Thiol functionalized QD; QY, quantum yield; SA, streptavidin; scFv, single chain Fv fragment; SMCC, succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; SPDP, N-succinimidyl-3-(2-pyridyldithio)propionate; sulfo-SMCC, sulfosuccinimidyl-4-(*N*-maleimidomethyl)

cyclohexane-1-carboxylate; sulfo-NHS, N-hydroxysulfosuccinimide; TCEP, Tris(2-carboxyethyl)phosphine hydrochloride; TGA, thioglycolic acid; TOPO, trioctylphosphine oxide.

1. Introduction

Colloidal quantum dots (QDs) are nanocrystals of a semiconducting material with diameters in the range of 1-20 nanometers [1, 2], constructed from elements of Group II (Zn, Cd, Hg)-VI (Se, S and Te), III-V and IV-VI of the periodic table. Until the last decade, most studies focused on II-VI QDs (CdSe or CdTe) but toxicity of Cd and the regulation [3] on its use restricts their implementation. Nowadays I-III-VI_2 semiconductor QDs (e.g. $CuInS_2$) are considered as one of the main alternatives of Cd-based QDs [4]. Due to QDs small size, the electrons are confined in a limited space, and when the radii of the semiconductor nanocrystal is smaller or equal than the excitation Bohr radius, there is quantization of the energy levels. This is responsible for the unique spectral characteristics and positions the QDs properties between the properties of atoms and bulk materials.

QDs represent a special class of inorganic luminophores and have certain advantages over conventional fluorescent dyes (e.g. rhodamine 6G) [5] (Figure 1). First, they have a high photostability due to a better resistance to chemical degradation and so they are less susceptible for photobleaching. Second, their broad absorption spectra make them ideal to excite photoluminescence (PL) with different colors of multiple QDs with a single excitation source. In addition, their narrow sharply-defined symmetrical emission spectra make it possible to combine different colored QDs without PL spectral overlap to perform multiplexing experiments [1, 2, 5-7]. Fourth, QDs have a much larger biochemically-accessible surface area compared to commonly used organic dyes, and subsequently facilitating the incorporation of multiple biomolecules [8].

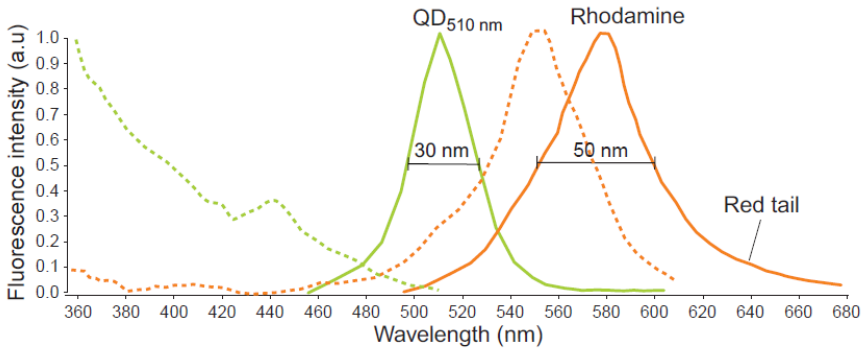

Figure 1: Comparison of the excitation and emission behavior of green CdSe QDs with Rhodamine 6G dye. The excitation spectrum (green dashed line) of a QD is very broad, whereas the spectrum of Rhodamine 6G (orange dashed line), is narrow. The QD emission spectrum (green line) is almost symmetric and much narrower in comparison with the Rhodamine 6G dye (orange line) [9].

The first colloidal QDs were synthesized in 1993 as 'core-only' CdSe QDs. It is well known that 'core-only' QDs are characterized by a low PL quantum yield (QY) (less than 10 % [5]), a limited resistance to photobleaching and their PL intensity is easily affected by charges and free radicals present in their environment. Later, in 1996, core/shell CdSe/ZnS nanoparticles were synthesized. The shell is responsible for an enhancement of the QY up to 18 % [1, 7] and photostability [6]. In addition, multishelling gives a better control on the shell quality and therefore the overall optical properties and stability of the QDs. Nowadays, QDs consist of three parts i.e. a core, shell and hydrophobic

capping layer. To obtain a high-ordered structure, which is important to obtain a high PL QY (gradient alloyed QDs: 27-61 % and homogenous alloyed QDs: 72 – 93% [10]), QDs are usually synthesized in high boiling organic solvents. Next, the QDs need to be made water-soluble (hydrophilization) for bio- and analytical applications.

2. Hydrophilization of QDs

PL QDs are one of the most promising nanoprobes for any kind of bio-application, such as chemical, biomedical and therapeutic labeling and imaging, cell targeting etc. But for these kinds of applications the particles should meet some requirements, e.g. QDs should be (i) stable in aqueous solutions over a wide pH and ionic strength range while (ii) maintaining their optical properties. Furthermore, QDs should (iii) have functional groups available for conjugation on their surface. The synthesis of QDs results in very hydrophobic nanoparticles that are only soluble in non-polar solvents. The highest PL QYs are usually observed in organic solvents, and introduction of QD into aqueous media is usually accompanied with a decrease of their luminescence QY [11]. Solubilization of QDs also makes a future conjugation to biomolecules, such as proteins, immunoglobulins (Igs), aptamers, oligonucleotides etc. possible. Over the years surface chemistry underwent a refinement which led to a decrease of non-specific binding and subsequently to great improvement of the QD specificity [5]. Eventually bioconjugation results in a multifunctional nanoparticle that combines the optical/electrochemical properties of QDs with the biological function of the biomolecule [1]. For example, in bio-imaging applications the QDs serve as imaging tag while the attached Igs may serve as the unique targeting agent through the specific antigen binding action [12].

There are three main strategies employed for hydrophilization of QDs (Figure 2). The first method involves ligand exchange, where the original hydrophobic coating (e.g. trioctylphosphine oxide (TOPO)) is removed and replaced with water-soluble bifunctional molecules in which one end is attached to the QD surface and the other end is hydrophilic and is available for bioconjugation. This approach is based on the application of polymers that contain some functional anchor groups, such as thiol, amine and carboxyl, which can passivate QDs more strongly than the original ligands. As other examples of linker molecules dithiothreitol (DTT), mercaptocarbonic acids, 2-aminoethanethiol, dihydrolipoic acid (DHLA), oligomeric phosphines, peptides, and crosslinked dendrons can be mentioned [13].

The second method is based on the well-known silica chemistry developed for coating metal nanoparticles (silanization). This strategy can be seen as a variant of ligand exchange strategy [14] but can be highlighted separately due to its recent wide use and prevalence. This strategy has a number of advantages over coating of nanoparticles with organic compounds. Coating of a single QD in a silica shell is a promising approach towards hydrophilization because the silica surface is non-toxic, chemically inert and optically transparent. A silica matrix can provide nanocrystals with an enhanced colloidal stability in polar solvents, chemical and physical shielding from direct environment, thus it protects the nanoparticles' surface from oxidation and other chemical processes. It also protects the outer environment from leaching of QD components (e.g. Cd). In addition, silica is a relatively biocompatible material and its surface can be easy functionalized, which makes QDs@SiO_2 suitable for bioconjugation [15, 16].

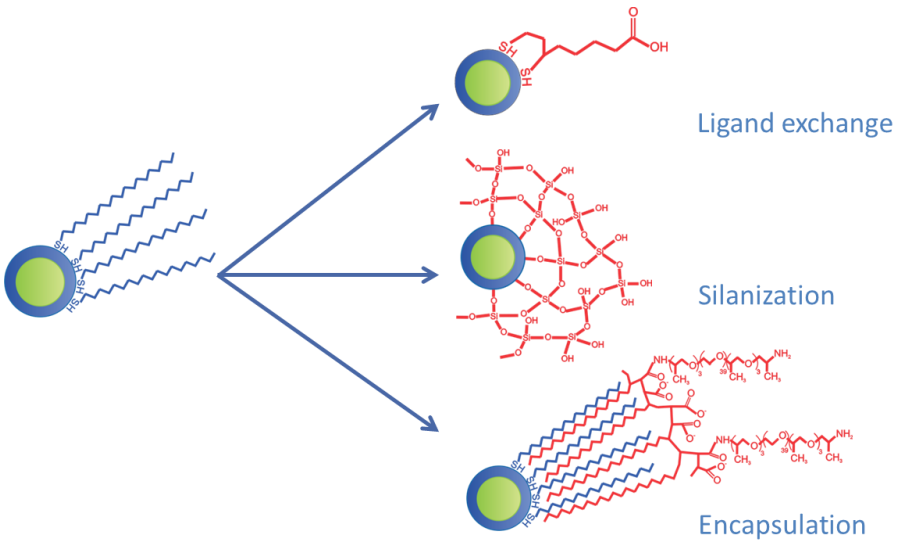

Figure 2: The main strategies for QD hydrophilization.

The third approach is based on encapsulation of the hydrophobic nanoparticles in different carrier vehicles e.g. amphiphilic polymers [17], polymeric microbeads [18] or liposomes [19]. Here the hydrophobic shell of QDs interacts with the original coating molecules through physical interaction, such as hydrophobic or electronic interactions. A number of amphiphilic copolymers (e.g. poly(maleic anhydride) copolymers [20]) and polyelectrolytes (poly-acrylamide [21], biopolymers like DNA [22]) were already synthesized to modify QDs surface [23]. Surfactant can also be used as encapsulant but is considered to be unstable in biological environment because of the relatively weak anchoring and does not protect the QDs from non-specific interactions. These problems were overcome by using amphiphilic polymers (e.g. polyethylene glycol (PEG) polymers [23]) which can have a strong interaction with the QD surface due to the presence of multiple hydrophobic and hydrophilic units [24]. Liposomes, which are spherical lipid vesicles, can also be used for encapsulation. Their hollow spherical structure and high loading capacity makes them attractive carriers for hydrophobic QDs [15, 19, 25]. It was shown that encapsulation of QDs into liposomes provides transfer of water-insoluble QD into aqueous media, facilitates their bioconjugation with proteins, minimizes non-specific interaction of water-soluble and water-insoluble QD with surface material, and amplifies the analytical signal [2]. A major drawback of liposomes is their instability *in vivo* and during storage as well as their high sensitivity towards external influences such as variation in temperature, pH and osmotic pressure [26], that can however be overcome through coverage or templating them with different materials, such as a polymer [27] or silica [25]. The most important is that QDs obtained through this approach have substantially increased hydrodynamic diameter which limit their application in bio-imaging and targeting [28].

In the case of QDs, hydrophilization directly affects the subsequent bioconjugation and future application. For example, some ligand exchange approaches can still allow access to the QD surface whereas the liposome or silica encapsulation completely precludes this. It affects the stickiness of the QDs which can lead to non-specific binding with different carriers, e.g. cells [6]. Further, size and hydrodynamic diameter of the final hydrophilic QD is highly dependent on which hydrophilization strategy is used. Ligand exchange provides QDs with a small hydrodynamic diameter but typically lower PL QY, while encapsulation results in larger sizes with higher QY [29]. The final size of water-soluble QDs determines their application. For example, QDs encapsulated with liposomes can be

easily used in a plate-format immunoassay, but cause a lot of difficulties in flow-through and lateral flow formats.

Nowadays, there are a lot of companies who offer hydrophobic and hydrophilic QDs of different composition, size and surface functionalization. Some examples of sources for hydrophobic QDs are Attonuclei (Nantes, France), CAN GmbH (Hamburg, Germany), MKnano (Toronto, Canada) and Nanoco group PLC (Manchester, England). Most of the commercially available QDs are composed by CdSe or CdS alloys and cover a wide range of wavelengths but CdS_xSe_{1-x} and cadmium-free QDs (e.g. InP and PdS) start to emerge more and more. A brief summary of commercially available hydrophilic QDs is shown in Table 1. Here, carboxyl –and amine functionalized QDs (QD-COOH and QD-NH_2) are mostly offered whether or not already conjugated with biomolecules (e.g. biotin or streptavidin (SA)). The major drawback of commercially available QDs is absence of any information concerning their exact composition and the composition of the hydrophilic shell. Usually only information about the nature of the active groups (amino, carboxyl etc.) on the surface of the particle is presented. This hampers subsequent bioconjugation of such QDs, as other chemical groups present on the surface can possibly be activated in the same conditions, can carry influential charges, or must be blocked to prevent any cross-reactions etc. In addition, such groups can influence the characterization of obtained target conjugates (such as electrophoresis, where charge is important).

Table 1: Commercially available hydrophilic QDs. Abbreviation: SA: streptavidin.

Company	QD (core/shell)	Emission range (nm)	Functionalization	Bioconjugation
Cytodiagnostics	CdS_xSe_{1-x}/ZnS	490-665	carboxyl, amine	/
	CdSeS/CdS/ZnS/ZnCdS	490-665	carboxyl	/
Thermo Fisher Scientific		525-800	carboxyl, amine (aldehyde, ketone)	SA, biotin, wheat germ agglutinin
	CdTe	510-780	carboxyl	/
Ocean NanoTech	CdSSe/ZnS, CdSe/ZnS	450-665	carboxyl, amine	/
	ZnCdTe	530-610	carboxyl	/
	CdSe	580	carboxyl	/
	CdSe/ZnS	450-665	?	Phenylboronic acid
Mesolight	ZnSe/ZnS	410 & 430	carboxyl, amine	/
	InP/ZnS	540-690	carboxyl, amine	/
	CdS/ZnS	420 -620	carboxyl, amine	/
	PbS	900-1300	carboxyl, amine	/
NN-Labs	CdSe/ZnS	530-660	carboxyl	/
	CuInS/ZnS, InP/ZnS	530-750	carboxyl	/

	Mn/ZnSe	600 & 620	carboxyl	/

3. Bioconjugation

There are two main approaches to immobilize biomolecules (packing) on the QD surface: covalent linking and non-covalent binding, including coupling directly to the QD surface or surface ligand coating the QD. Non-covalent binding is determined by hydrophobic, electrostatic, or affinity interactions between biomolecules (e.g. Ig) and the QD surface; covalent linkage is obtained via different bioconjugation chemistry using activated functional groups at the surface of QDs [29, 30]. The ability to conjugate QDs to biomolecules was first performed by Bruchez [31] and Chan [32] in 1998 and has since been greatly exploited in a variety of imaging, immunoassays, and DNA sequencing techniques.

In general, the use of (strept)avidin binding to the biotin molecule, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) condensation as well as thiol-to-maleimide coupling strategies are more common due to the fact that these protocols have been well established and are ubiquitous in biology [33]. These probes are generally constructed from QDs encapsulated in amphiphilic polymers or micelles, which has been a popular route to obtain biocompatibility. While this design facilitates good conjugate stability and enables targeting, it suffers from high multivalency and a large hydrodynamic diameter (~25-40 nm), depending on the particular surface coating and conjugated biomolecules. Some efforts have been made to design QD probes with reduced size and valency [34]. Table 2 summarizes the advantages and drawbacks of non-covalent -and dative conjugation strategies together with the applications in which these binding strategies should not be used. The advantages and drawbacks for covalent coupling procedures are summarized in Table 3.

Table 2: Summary advantages and drawbacks non-covalent and dative conjugation strategies. In addition, per binding strategy the reason why (between brackets) and the application itself in which the conjugation strategies should not/ cannot be used are mentioned. Abbreviations: FRET: fluorescence resonance energy transfer, His: histidine, Ig: immunoglobulin, Ni-NTA: nickelnitrilotriacetic acid, SA: streptavidin, QD: quantum dot.

Conjugation strategy		Advantages	Drawbacks	No preference applications (reason)
Electrostatic		• Easy • Fast • No need crosslinkers • Less aggregation and crosslinking QDs	• Chance activity biomolecules compromised • No control orientation • No biomolecule/QD ratio control • Less stable interaction • Non-specific interactions	• Cellular research (stability)
High affinity secondary interactions	**SA-biotin**	• Stable interaction • Biotinylated monomer for polymer coated QDs • QDs-SA commercially available	• Large size bioconjugates • Variety orientations • Chance over biotinylation	• FRET (size) • Cellular research (size) • *In vivo* applications (biotin common in mammalian tissue)

Dative chemistry	**NTA-Ni^{2+}-His**	• Control orientation • Low cost • Compact size	• Less stable interaction	• Immunochemical staining (stability)
	Barnase-barstar interaction	• Small proteins • No covalent modification protein • Monomeric protein		
	Ig-ligand	• No modification biomolecule • Ease purification • No non-specific & null combinations • Control orientation • Stable interaction	• Large size bioconjugates • Engineered proteins • Heterogeneous protein/QD ratio	
	Metal affinity coordination	• Little or no aggregation and crosslinking • Control valence • Control orientation • Applicable on coated QDs (small molecules) • Ease purification • Stable interation (in case of polyHis)	• Less stable interaction • Engineered proteins	• Tissue imaging (stability – competition) • Drug delivery (stability – competition, unless it is intention)
	Thiol interactions	• Thiolated proteins can act as solubilizing ligands	• Less stable interaction • Non-specific interaction • Biomolecule should contain thiol group • Direct access QD surface • Sometimes extra passivation QD necessary	

Table 3: Summary advantages and drawbacks covalent conjugation procedures. Abbreviations: CDI: N, N'-carbonyldiimidazole, EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, QY: quantum yield, TCEP: Tris(2-carboxyethyl)phosphine hydrochloride.

Functionalized QD	Attached biomolecule	Advantages	Drawbacks
QDs-COOH	**Biomolecule-NH_2**	• Simple • Easy • Small size bioconjugates • Low yield (EDC alone) • Glutaraldehyde: higher QY	• Activation carboxyl group • EDC: low QY (pH reasons) • High amount EDC needed (hydrolysis) • No control valence • No control orientation • Aggregation and crosslinking
	Carbohydrate hydroxyls	• Built in spacer • Away from binding site	• Activation by another compound • Side/cross-reactions
QDs-NH_2	**Biomolecule-SH**	• Heterobifunctional crosslinkers • More selective • Less crosslinking • Homogeneous orientation	• Hydrolysis • Ig reduction

	Carbohydrate hydroxyls **Biomolecule-NH_2**	• More selective	• Uncontrollability • Aggregation • Crosslinking (non-specific interaction) • Not reproducible
QDs-SH		• More selective • Direct solubilisation QDs possible • More stable in cellular media (vs disulfide bond) • Spectrophotometrical follow-up	• Molecule should contain thiol group • Large size bioconjugates • Side reactions (pH dependent) • Aggregation • Instability QD solution by reducing agents (e.g. TCEP)
QDs-epoxide		• Small size bioconjugates • No crosslinker • Reacts with different functional groups • Strong emission QD after conjugation	• Aggregation • Hydrolysis
QDs-OH		• Stable bioconjugates • Less aggregation • Less non-specific binding • Photostability • Strong emission	• Slow coupling (CDI) • Alkaline conditions (CDI)
QDs-aldehyde		• No cross-reaction • Efficient coupling • Chemoselective • Biomolecule/QD ratio control • Use of UV traceable markers	• Catalyst necessary

3.1. Packing of biomolecules on QDs

The amount and orientation of biomolecules that can be attached to the QD surface depends on a combination of factors, like the size of the QDs, properties of the QD surface, nature of the biomolecule (i.e. globular protein versus long DNA or rigid peptide strands), steric reasons and the used bioconjugation technique. Comparing literature in order to classify different bioconjugation techniques according to the amount of immobilized biomolecules is difficult because, often, several mechanisms of binding or packing are used and usually QD surface modification and hydrophilization are simultaneously realized (Table 4).

For steric reasons only 2 to 5 molecules of a 100 kDa protein can be attached to a 5 nm QD, which is similar to the number of protein molecules that can be attached to a 5 nm colloidal gold particle [32] but in general it is suggested that an average of 2-10 Igs can be conjugated per QD. As already mentioned, the hydrophilic coating also determines the bioconjugation as it delivers the necessary active groups and should also be taken into consideration. Chan and co-workers also report immobilization of 2-5 protein molecules to a single 4 nm QD. Further, they mention that 50 or more small molecules (such as oligonucleotides or peptides) can be conjugated to a 4 nm QD [35, 36].

Research has been performed on the immobilization of whole and reduced Igs on QDs by covalent strategy. As steric hindrance of half bodies of Ig is less than the whole bodies of Igs it was expected and confirmed that more reduced Igs were able to bind to the QDs. In addition, when reduced Igs were coupled they received a more various QD/Ig ratio (1-2.2 ratio vs. 1.3) (Table 4) [37]. Medintz's group also confirmed that when larger protein molecules (17-44 kDa) were conjugated by metal affinity coordination steric hindrance is the major packing constraint. In contrast, for smaller peptides (< 2.2 kDa), the number of available QD binding sites determines the maximum loading achievable and this also indirectly reflects the level of QD surface coverage [38]. The shape of the protein (e.g. monomeric vs rigid bridge-shaped dimeric structures) itself also influences the saturation ratio [39]. Banerjee et al. (2015) [28] investigated the effect of the length and the use of double or single stranded DNA. They observed that the coupling efficiency depends on the DNA length, the smaller the DNA strand the more DNA could be conjugated to the QDs with preservation of the photophysical and structural properties of QDs and DNA, respectively. The conjugation of double stranded DNA was not as efficient as single stranded DNA of the same length.

The amount of immobilized biomolecules is not only determined by steric hindrance but also by the chosen bioconjugation strategy. For example, by using a covalent strategy (DTT / succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC)) the number of functional Igs bound to QDs was on average much less than in the case of a non-covalent (SA-avidin) strategy. This suggests that the non-covalent strategy results in a better packing of biomolecules on the QD surface. Important to mention is that in the case of the SA-biotin system the entire Ig was conjugated to the QD; it was not reduced into light chain and heavy chain [40]. Song & Chan (2011) [41] used carbodiimide chemistry for the coupling of IgG to amphiphilic polymer coated CdSe/ZnS and investigated the influence of different EDC ratios. They were able to bind 3 or more Igs to QDs but an excess of EDC resulted in massive aggregation. However, in contrast to what is mentioned in literature they observed a decrease in avidity when more than 3 proteins were coupled to a single QD.

Pons et al. (2006) [42] studied the distribution of histidine (His_n)-tagged proteins per QD in a solution of QD-protein conjugates with a given nominal protein-to-QD ratio below saturation of the QD surface. They confirmed that in this situation the process follows a Poisson distribution. This means that the number of biomolecules coupled per QD will not be uniform across an ensemble but rather a statistical function of the average stoichiometry in the ensemble. For a conjugate with a nominal valence *N*, the fraction of QDs conjugates conjugated to exactly *n* biomolecules is then theoretically given by

$$p(N,n) = N^n/n!\ \exp(-N) \quad (1)$$

Where *N* is the average stoichiometry, *n* is the exact number of biomolecules bound to a QD, and *p* is the relative fraction of each subpopulation. In practice this means that conjugates which are prepared with a low average stoichiometry will have a non-trivial population of unconjugated QDs unless special steps are taken to isolate the sub-population with the desired conjugate valence (e.g. gel electrophoresis). When the average conjugate valence exceeds 4 biomolecules per QD the effect of the Poisson distribution is minimized and can be neglected [42, 43].

It can also happen the other way around, where more QDs are immobilized on a biomolecule to increase the signal output. An often used biomolecule for this purpose is apoferritin, which consists of 24 subunits, each of which is genetically-modified to express His_6-tag and protein G. The His_6-tag

can be used to immobilize nickelnitrilotriacetic acid (Ni-NTA)-functionalized QDs and protein G can be used to bind the Fc-region of a specific Ig. This results in an increase of sensitivity and specificity. Research showed that approximately 5 to 6 QDs can be attached per apoferritin unit, which results in a 4.5-fold increase in fluorescence intensity compared to a single QD [44, 45].

Table 4: Overview of biomolecules packing on QD surface arranged by bioconjugation strategy. The molecule/QD ratio mentioned is the actual/estimated amount of molecules that were able to couple to the QD and not the saturation ratio. Abbreviations: bp: base pair, DDT: dithiothreitol, EDC: 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, His: histidine, His-Ni-NTA: Histidine-nickelnitrilotriacetic acid, Ig: immunoglobulin, MBP: maltose-binding protein, sulfo-NHS: N-hydroxysulfosuccinimide, SA: streptavidin, SMCC: succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, TCEP: Tris(2-carboxyethyl)phosphine hydrochloride.

QD size	Bioconjugation strategy	Coupled molecule	molecule/QD ratio	reference
5.7-5.8 nm	EDC	SA	5-15	[46]
12-14 nm	EDC/sulfo-NHS	Ig	0.84	[47]
Not mentioned	EDC/sulfo-NHS	IgG	1-3	[41]
7 ± 0.3 nm	EDC/sulfo-NHS	HER2 Ig	1.3 ± 0.2	[37]
18-20 nm	DCC/NHS	Aflatoxin B_1	6.8	[47]
7 ± 0.3 nm	SMCC	Reduced anti-HER2 Ig	Various ratio (from 1.0-2.2)	[37]
7 ± 0.3 nm	Iminothiolane/SMCC	HER2 Ig	1.3 ± 0.2	[37]
7.7 ± 0.8 nm	SMCC/TCEP	15 bp single stranded DNA	11.5 ± 4.0	[28]
7.7 ± 0.8 nm	SMCC/TCEP	45 bp single stranded DNA	4.3 ± 1.0	[28]
7.7 ± 0.8 nm	SMCC/TCEP	15 bp double stranded DNA	4.7 ± 1.2	[28]
560 nm emitting	His_5 coordination	MBP (44 kDa)	12-15	[48]
530 nm emitting	His_5 coordination	MBP (44 kDa)	10	[48]
± 6 nm	His_5 coordination	MBP (44 kDa)	12 ± 2	[38]
6 nm	His_5 coordination	MBP (44 kDa)	12-15	[48]
Core + shell = 2.8 mm	His_6 coordination	Peptide (4-Formylbenzoyl-Amino-hexanoyl-$Pro_9Gly_2His_6$)	11.0	[49]
± 6 nm	His_6 coordination	Myoglobine (17 kDa)	30 ± 5	[38]
± 6 nm	His_6 coordination	mCherry (27 kDa)	20 ± 4	[38]

2.5-3.5 nm	His_6 coordination	peptide-DNA (29 or 30 bp)	12	[50]
± 6 nm	$His_{4\ or\ 8}$ coordination	Peptide 1.58 kDa and 2.13 kDa	50 ± 10	[38]
10.32 nm	His-Ni-NTA	apoferritin	1/5-6	[44, 45]

3.2. Non-covalent binding

Non-covalent attachment of biomolecules to the QD surface (Table 2) is mainly based on two types of QD surface-biomolecule interaction: electrostatic interaction between oppositely charged molecules and high affinity secondary interactions (e.g. biotin/(strept)avidin). Simple non-covalent adsorption of the biomolecules to the QD surface is the least demanding approach but the activity of the biomolecules can be compromised and there is no control over the orientation and valency of biomolecules. In most cases, non-covalent attachment arises by comparatively weak coordination binding, and consequently the stability is quite sensitive to the QD and biomolecule concentrations during preparation and in their application. By using high affinity secondary interactions the biomolecules can be bound in a proper orientation.

3.2.1. Bioconjugation by electrostatic interaction

QDs possess a large surface area that can be utilized for non-specific adsorption of large molecules such as polymers, long chain organic molecules, proteins, enzymes and nucleic acids. This strategy results in non-selective binding of molecules and does not depend upon any functional attachment [12].The simplest and most widely used non-covalent bioconjugation approach is electrostatic attachment because it requires no chemical reactions per se. The principle behind electrostatic interaction between QDs and small molecules or biomolecules is the attraction between oppositely charged species [29]. Since this bioconjugation often requires only stoichiometric mixing of the two components, they are typically referred to as self-assembly. A well-known method of electrostatic coupling of proteins with QDs is self-assembly on negatively charged QDs (e.g. by presence of carboxyl groups) due to the natural positive surface charge of a protein. Relevant protein molecules can also be engineered to express positively charged domains, such as the leucine zipper [51] at the C-terminus, on their surface [12, 30]. Another option is the interaction of positively charged His residues (in polyHis-tag motifs of proteins) with QDs-COOH. This method significantly reduces the overall size of the bioconjugates. The size of peptide-QD conjugates can also be decreased by using only the relevant fragments of Igs or short peptides with attached His-tags that adequately mimic the behavior of a full-size protein [30].

Although, the non-covalent concept is quite simple and rapid, without the need for adding reagents it comes with several limitations. In most cases, non-covalent attachment is based on comparatively weak coordination binding especially in the presence of competitive molecules (e.g. when the QD-bioconjugate is present in cellular environment). Factors such as ionic strength, pH, and the type and magnitude of charge play an important role in obtaining the desired conjugates. The stability of the QD-bioconjugates can be improved by incorporating more charged groups at the QD surface, which also allows to work with lower concentration of biomolecules [29, 30]. Other reported drawbacks are

the difficulty to quantify the non-specific interaction and thus the 'QDs to protein ratio' and orientation of attached biomolecules cannot be controlled [12]. It was also reported that association of proteins with a charged surface of nanoparticles influences the protein secondary structure, which may alter their enzymatic activity [30].

Non-covalent binding, however, has the advantage that it does not rely on a particular linking chemistry and the constraints that this may bring such as aggregation, crosslinking etc [8]. Covalent attachment strategies are more popular when a strong bonding and correct orientation of the biomolecules conjugates at the surface of QDs are necessary [12].

3.2.2. Bioconjugation based on high affinity secondary interactions

Secondary interactions between functional groups covalently attached to the QD surface and biomolecules are another class of non-covalent interaction. Among high affinity interactions, like DNA interactions, receptor-ligand or enzyme-substrate interactions, biotin-avidin (or nowadays the more often used SA) interaction where the QD and biomolecule are functionalized with biotin or (strept)avidin is most used. Bioconjugation of QDs via biotin-avidin chemistry is ubiquitous but is less popular as EDC-based covalent conjugation chemistry because it often produces large size bioconjugates, which can limit their utility in targeted applications such as fluorescence resonance energy transfer (FRET) and cellular research. Other approaches can utilize Ig affinity for a ligand attached to the QD or vice versa, along with other enzyme-substrate or receptor-ligand interactions [29, 43, 52].

Biotin-(strept)avidin interaction. The number of publications incorporating biotin-(strept)avidin chemistry for bioconjugation of QDs is still increasing. The biotin-avidin complex is, due to the high affinity between avidin and biotin (dissociation constant (K_D) of 10^{-15} M^{-1}) [53] and their well-understood interactions, a very popular method of attaching biomolecules, such as Igs or DNA, onto the surface of a QD. In this case, the interaction is, in essence, functioning as an intermediate linking technique. Avidin, per se, has the tendency to display non-specific binding due to its basic isoelectric point (pI, the point where the molecule carries no net charge), and carbohydrate content. This leads to severe QD-QD aggregation due to the highly positive charge of avidin at physiological pH resulting in clusters of QDs connected electrostatically through avidin bridges [54]. This issue was overcome by recombinant or chemical deglycosylating avidin without affecting its biotin affinity. Removal of the carbohydrate functionality together with a reduction in the pI (to ~6.3) has largely ameliorated non-specific binding issues. As an alternative, the analogous protein SA, isolated from the bacteria *Streptomyces avidinii*, is a homologous tetrametric biotin binding protein displaying similar affinity to avidin. SA is not a glycoprotein and the lack of high carbohydrate content, combined with a much lower pI of 5-6, which makes SA neutral at physiological pH also results in a lower non-specific binding. There are now a number of genetically engineered avidin and SA derivatives commercially available [29, 43]. Biotin is a relatively simple water-soluble organic compound, also known as vitamin B_7 or vitamin H. There are a variety of biotin derivatives that make biotinylation of QDs and biological molecules rather straightforward. These are synthesized to display amine, thiol, carboxyl, azide, and other functional groups that facilitate conjugation. An extensive range of very effective reactive biotin reagents are available such as maleimide, NHS ester, or other adducts, which are intended to "biotinylate" their target group on biologicals, particularly proteins and Igs, along with nanoparticles. It should be noted that, since endogenous biotin is common in mamalian tissues and

may interfere with biotinylated conjugates, SA-biotin systems may be unsuitable for *in vivo* applications [30].

There are two principle methods of bioconjugation using biotin-(strept)avidin chemistry. The first, and probably most common, approach involves SA-functionalized QDs being mixed with biotinylated biomolecules. It is important to consider this will eventually result in to a variety of orientations of the biomolecules. In the case of Ig (or antigen) this can result in non-epitaxial conjugation which will lead to a decrease of sensitivity and specificity of the bio-detection [29]. Biotinylation of biomolecules usually involves chemical preparation with an amine-, thiol- or carboxyl-reactive biotin reagent or, alternatively, the molecule can be recombinantly modified to display biotin acceptor peptide sequences [43]. An important issue to consider when conjugating biotin to groups such as the amines distributed around a protein, is that control over the labeling site and ratio is hard, given the abundance of targets. In case of DNA or peptides biotin groups can be introduced during synthesis. The second approach is often used in conjunction with polymer-covered QDs, where researchers take advantage of common organic synthetic procedures to biotinylate the "monomer" unit prior to its incorporation into the polymer covered QD. In this case, biotin is preferred over SA labeling because the small size of the biotin moiety means that it can be chemically inserted into nascent peptides or DNA during synthesis at almost any site of choice for subsequent QD attachment.

In practice, biotinylated QDs will either be directly linked to a SA-conjugate or to another biotinylated molecule and by means of crosslinking via an (strept)avidin "bridge". Since each (strept)avidin protein is an obligate tetramer it can bind up to four biotin moieties, so care must be taken not to over biotinylate the biomolecule of interest, since this can induce crosslinking of the QDs and formation of undesired conjugates [43]. Overall, the detailed fundamental understanding of the (strept)avidin-biotin interaction, its wide utility, and the extensive library and ongoing evolution of reagents are responsible for the continued use of (strept)avidin-biotin chemistry for QD bioconjugation in the foreseeable future [29]. Biotinylated QDs are applicable in immunochemistry based on the widely used biotin-SA amplification approach [55]. Another application is the development of aptasensors [56].

Histidine-nickelnitrilotriacetic acid interaction. The interaction between His-tagged biomolecules and Ni-NTA is also a well-known high affinity secondary interaction and is consequently used for QD bioconjugation. The strong interaction (NTA-Ni^{2+}-His) has a K_D of~10^{-13} [57]. In this case, the nitriloacetic acid group is covalently linked to the QD encapsulation polymer whereas His-tagged biomolecules bind to nickel ions (Ni^{2+}) by chelation. In comparison with biotin–avidin binding, this ''His-tag'' method has several advantages such as a controlled orientation of the binding ligand (a His-tag can be conveniently fused to proteins and peptides at a particular site), compact overall probe sizes and low production costs. Although the stability of the His– Ni^{2+} attachment could be a problem under the harsh conditions of immunohistochemical staining, previous research has shown that the interaction of His_6 with Ni-NTA is virtually unaffected by high ionic strength (up to 1 M), non-ionic detergents, organic solvents, ethanol or glycerol up to 30%, reducing agents (b-mercaptoethanol) or highly denaturing conditions (urea or guanidine hydrochloride) [58]. This strategy has also been used to develop a QD-probe for performing Western blot analysis with a shorter analysis time and a better sensitivity when compared with the classical Western blot [59].

QDs can be functionalized with Ni-NTA during water-solubilization of the hydrophobic QDs and can interact with apoferritin, which is genetically-modified to express His_6 and protein G [44].

Barnase-barstar interaction. A less known strategy is based on the barnase-barstar system, initially developed for Ig multimerization, used for bioconjugation along with the SA-biotin system. Barnase and barstar are small proteins (12 and 10 kDa, respectively) with an extremely high mutual affinity (K_D of ~10^{-14}) comparable with the affinity of SA-biotin interaction (K_D of ~10^{-15}). Barnase is a secreted ribonuclease from *Bacillus amyloliquefaciens*. Barstar is a cytoplasmatic barnase inhibitor with which the host protects itself. Unlike biotin, which is not a peptide, barnase and barstar can be fused with proteins and Igs by genetic engineering methods. Both can be efficiently expressed in bacteria in recombinant form [30]. Unlike the biotin-avidin system, this system does not require covalent modifications of any of the proteins, and both partners are monomeric. The 3D structure of the barnase-barstar complex shows that the N and C termini of both proteins are localized away from the dimerization interface. Therefore, all four termini are accessible and available for fusions. When they are attached to a single chain Fv fragment (scFv) via a hinge region, they serve as building blocks for multi-mini Igs that mimic the rational and segmental flexibility of the natural Ig binding arms. By fusing more than one barnase (or barstar) in series, complexes of higher valency can be created, as each one will complex with a partner. The extremely specific and strong association of barnase and barstar makes it possible to obtain a precise 1:1 ratio of partners [60]. Complexes of mercaptoacetic acid (MAA) coated QDs with recombinant antitumor Igs (with EDC as crosslinker) have been constructed using the barnase-barstar system by Deyev's group. They also designed a scFv-(barnase-barnase) fusion protein (termed 'dibarnase'), in which two barnase units were directly fused head-to-tail without a linker. This module allowed binding of the scFv Ig to the QDs [61]. Taken together, data has shown that the barnase-barstar system constitutes a useful tool for the design of oligomeric proteins for basic research as well as for imaging [62] and therapeutic applications [60].

Antibody-ligand interaction. Ig affinity for a ligand attached to a QD or vice versa [29, 43, 52] can also be utilized has high affinity secondary interaction. Molecular adaptor proteins (proteins A, G and A/G) can be attached to the QD surface and are capable of binding different types of Igs. The conjugation of these proteins to a QD can happen through a leucine zipper or His-tags. It is also the easiest way to construct oriented functional QD-Ig complexes and does not require any modification of Igs. QD complexes with Igs may be prepared using a more complex linker consisting of a molecular adaptor protein and a second protein used as a purification tool. Protein G modified with the leucine zipper acts as a molecular adaptor to connect a QD with Igs, whereas maltose-binding protein (MBP), also connected with the QD via the leucine zipper, serves as a purification tool for separating the QD-IgG conjugate from excess IgG by means of affinity chromatography [30]. The labels can be connected to the Igs through protein G- a "flexible bridge" – without occupying the Fab domain and influencing the bio-specificity of the Ig. Zeng et al. (2009) [63] and Gilroy et al. (2010) [64] covalently coupled QDs-COOH with protein G and protein A/G, respectively, by using EDC/ N-hydroxysulfosuccinimide (sulfo-NHS) to make a QD-protein G and A/G complex which was then bound to Igs. Hereby, non-specific and null combinations were avoided, resulting in an increased specificity and sensitivity of the immunoassay. Gilroy et al. (2010) [64] were one of the first able to use recombinantly engineered variants of the above mentioned proteins, that could be stable covalently conjugated. The chimeric protein A/G contains the IgG binding domains of both Staphylococcal protein A and Streptococcal G, which contains four Fc binding domains from protein A and two from protein G. Because of these combinations the protein A/G is a powerful tool in the

binding of IgG from multiple species. The probes could be used in a multiplexed Western blot to detect simultaneously four proteins in the presence of cell lysate, overcoming issues surrounding non-specific binding of conjugates.

3.3. Dative chemistry

Direct conjugation of a biomolecule to a QD surface is usually driven by dative bonds, while attachment to ligands is accomplished with covalent bonds. QD-bioconjugates can be prepared based on two types of dative interactions: metal-affinity coordination and thiol interactions (Table 2). Dative or coordinate covalent bonds result when two electrons in the bond originate from a single atom and are characterized by their longer bond lengths, lower energies, and greater polarity than covalent bonds. Dative bonds are not as strong as true covalent linkages and can be compromised by changes in pH, oxidation, and displacement by other similar molecules. For example monodentate thiol interactions with QD surfaces are characterized by very dynamic off-rates that can limit long-term conjugate stability. These types of bonds can be strengthened by increasing the number of interactions (i.e., multivalency) [29]. An illustration of the pH dependence of imidazole groups' stability is given by Petryayeva et al. (2012) [65].

Metal affinity can be realized between His motifs and divalent transition metals, especially Zn(II) present on the QD surface. Unlike covalent strategies, this method usually results in protein-QD dispersions with little or no particle aggregation [30]. His-tag typically contains six imidazole rings connected via amino acid peptide linkages. It has been demonstrated that increasing the number of His amino acids within a tag can increase the affinity to QDs [52]. Binding of His-tag modified biomolecules proceeds virtually quantitatively, eliminating the typical need for additional reagents and subsequent purification steps that arise when using other conjugation methods [65]. Despite the relatively high affinity between metal and His-tag, QD-protein assemblies are still prone to displacement by competitive ligands, such as imidazole, glutathione (GSH), and His-tag peptides. As biological fluids often contain metal-binding molecules and proteins, ligand displacement will result in mixed ligands on QDs, and hamper their use in tissue imaging or drug delivery [39]. Although His_n-QD surface interactions have been postulated for some time, the first description in literature was in 2005 by Medintz and co-workers [52, 65]. His_n-QD interactions also permit control over QD bioconjugation valency by an approximate one-to-one correlation with molar stoichiometries. It is important to note that this correlation is meant to apply to the average valency; the actual distribution within a particular configuration would follow a Poisson distribution process, and this is especially true at lower ratios. Interestingly, His_6-driven metal affinity is also applicable to many different QD preparations despite the fact that they are surface-functionalized with encapsulating polymers (e.g. lipid/PEG ligand). Large proteins tend to be sterically precluded from the QD surface when the ligands are bulky such as in the case of PEGylated molecules. Smaller, far-less bulky peptides and DNA with their more linear structures usually do not encounter this issue [29]. The use of His_n motifs has several advantages for assembly to QDs. His_n motifs can be used to facilitate purification of recombinant proteins with Ni-NTA resin and does not suffer from crosslinking because they provide a single point of attachment. By using this chemistry, control over the biomolecular orientation on the QD is achieved and when used in living systems there is no interference with any native biochemical process, since they are not normally found in natural proteins. When incorporated in proteins, there is no disturbing of the proteins function because of their small size

[29]. Mattousi's group demonstrated the chemical attachment of a thiol-reactive His_6 peptidic linker to thiolated DNA oligonucleotides to enable their self-assembly onto QDs via disulfide exchange [52].

Thiol-displaying molecules can also be datively coordinated to the surface of QDs, presumably by interactions with available zinc and sulfur moieties on the QD surface. However, two issues need to be taken into account when using this approach namely the biomolecule (e.g. protein) needs to display available and reduced thiols (e.g. non-dimerized cysteines) on its periphery and have direct access to the QD surface without hindrance from the capping ligands. In the case of proteins, the thiolated proteins could then act as surface solubilizing caps or ligands for the QDs. In 2012, Mansur et al. reported that the interaction between albumin proteins and nanoparticles does not exclusively occur via thiol groups present in cysteine amino acids, like most reports assume. His group reported that some other amino acids have also paramount importance on these interactions, such as aspartic acid, glutamic acid and His [66]. The influence of the capping ligands was investigated by comparing the interaction of bovine serum albumin (BSA) and CdTe QDs modified with 3-mercaptopropionic acid (MPA), thioglycolic acid (TGA) and GSH. Also different conjugation techniques, electrostatic and covalent by using EDC and NHS, were compared. When BSA and QDs were conjugated on an electrostatic manner the results indicated that the number of amine groups can strongly influence the interactions between BSA and QDs capped with GSH. In case of a covalent conjugation, QDs can bind to BSA but GSH-QDs gave the best result due by the presence of higher number of amine groups in GSH (3 groups) compared to MPA and TGA [67]. Steric hindrance is less of an issue when smaller peptides and DNA are used. Thiolated peptides or DNA can even be used to cap the QDs but even with smaller biomolecules problems can be encountered. QDs capped with MPA, GSH were successful conjugated with a bidentate thiol-modified oligonucleotide probe. Whereas the same strategy with QDs capped with DHLA ligands gave problems. It is interesting to note that, while MPA coated QDs exhibited strong non-specific adsorption and required passivation with denatured BSA (dBSA), as described previously, GSH coated QDs exhibited minimal to non-detectable non-specific interactions with oligonucleotides. The partially zwitterionic character of the small peptide used as a coating ligand could significantly minimize hydrogen bonding that has been reported for MPA ligands [65]. To increase the long-term conjugate stability an alternate approach relied on growing an amorphous silica shell around water-soluble CdTe core-only QDs, followed by modification of the shell with PEG and thiol-terminated linkers, which could then be conjugated to Igs. Thiolated peptides have been applied for direct cap exchange onto the QD, thus providing both solubility and, if needed, intrinsic biological utility. Overall, the use of dative thiol bonding for QD bioconjugation has diminished in recent years with the advent of newer and more versatile chemistries [29, 43].

3.4. Covalent binding

Covalent conjugation between QDs and molecules involves the reaction of one functional group with another, resulting in formation of a covalent bond. For QDs, the used hydrophilization strategy determines which functional groups are present on the QD surface and this, subsequently, determines the covalent binding strategy. Covalent binding can be realized by use of functional crosslinkers. These last ones can be defined as bifunctional (or multifunctional) reactive molecules meant to join two (or more) molecular entities together; but they can also serve to introduce a new functional group, for example converting a QD-NH_2 to also display free thiols. Crosslinkers can differ in the amount of additional atoms (linker length) they add after bioconjugation and the nature of

their functional groups. The smallest available crosslinkers for bioconjugation are so-called zero-length crosslinkers (e.g. EDC and N,N'-Dicyclohexyl carbodiimide (DCC)) (Figure 3). These compounds mediate the conjugation of two molecules by adding no additional atoms. Other popular crosslinking reagents used for modification and conjugation of macromolecules are homobifunctional crosslinkers (e.g. dithiobis(succinimidylpropionate) (DSP)) (Figure 3), which display the same functional group on both ends of an alkyl spacer. In practice, these reagents connect two molecules by reacting with the same functional group on these molecules (Figure 4). A disadvantage of these crosslinkers is the potential of creating a range of conjugates. These conjugates can be the result of intramolecularly reaction of a protein with other functional groups, an activated protein that also react with a second protein or another molecule. To avoid or reduce this problem two-step procedures are developed when using homobifunctional groups [29, 68].

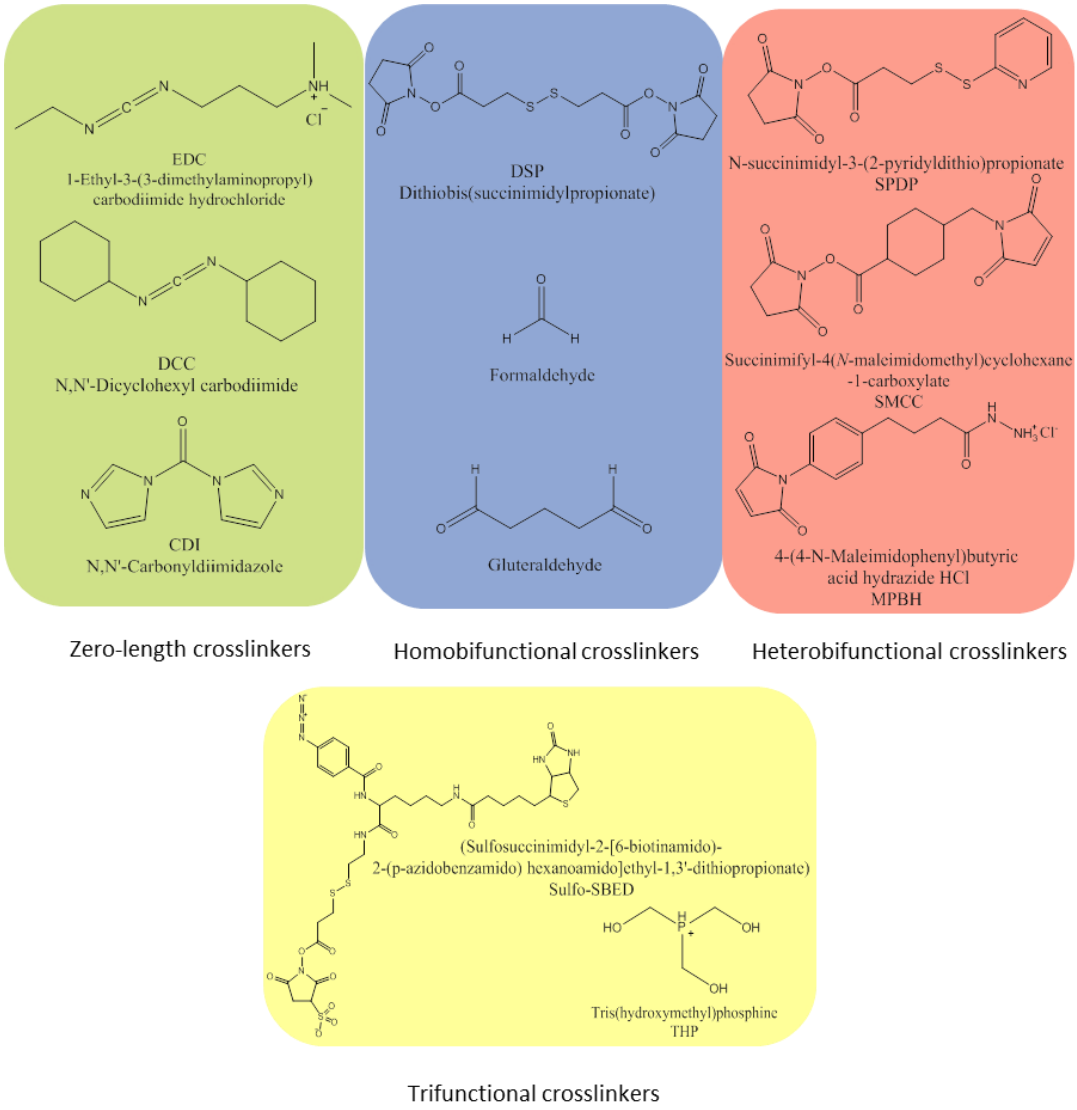

Figure 3: Bioconjugate reagents categorized as zero-length crosslinkers; homo-, hetero-, and trifunctional crosslinkers.

Heterobifunctional crosslinkers (e.g. N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP)) contain two different reactive groups, allowing coupling of two different functional targets (Figure 3). For example, one part of a crosslinker may contain an amine-reactive group, while another part may consist of a thiol-reactive group (Figure 5). Heterobifunctional crosslinkers give a better control over

the conjugation because the reaction will take place in specific parts of the target molecules. They are usually used in a two- or three-step process that limits the degree of polymerization often obtained by using homobifunctional crosslinkers. However, two-step procedures increase the chance of hydrolysis of the activated intermediate before addition of the second molecule. It is important to note that since amines and thiols are both good nucleophiles, amine-thiol crosslinking is usually done in two steps, starting with conjugation to the amine [29, 68].

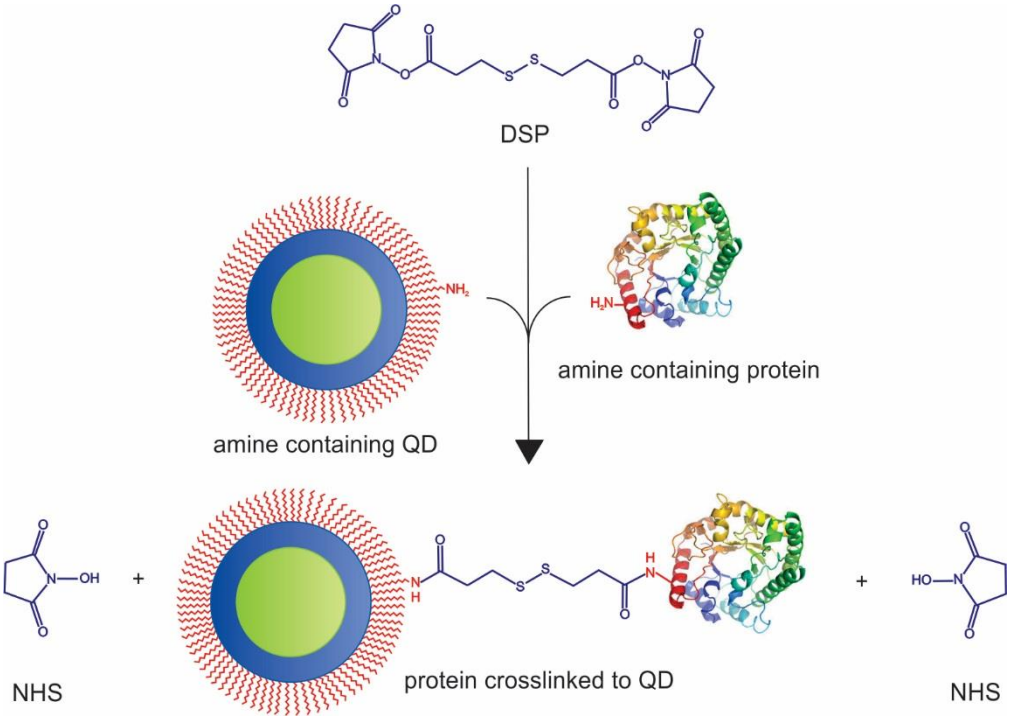

Figure 4: Conjugation of an amine containing QD to a protein by using the homobifunctional crosslinker DSP.

Trifunctional crosslinkers are a relatively small group of bioconjugation reagents (Figure 3). They possess three different reactive groups but are far more complicated to design. Nevertheless, given the long-term needs of nanoparticle bioconjugation chemistry, they may also have the most to offer for assembling far more complex multifunctional biomolecular structures [69].

In conclusion, a big variety of homo-, hetero-, and trifunctional crosslinkers are available where the length and type of the cross-bridge differs (e.g. long chain SPDP (LC-SPDP)). Different linker lengths can give a level of control over separation distances and orientation between crosslinked molecules, which may help optimize subsequent binding interactions [29, 68]. In general, it is advisable that when QDs are coated with molecules containing different functional groups they should be coupled with relative simple biomolecules. To prevent all possible side reactions it is better to activate first the biomolecules followed by conjugation to the QDs.

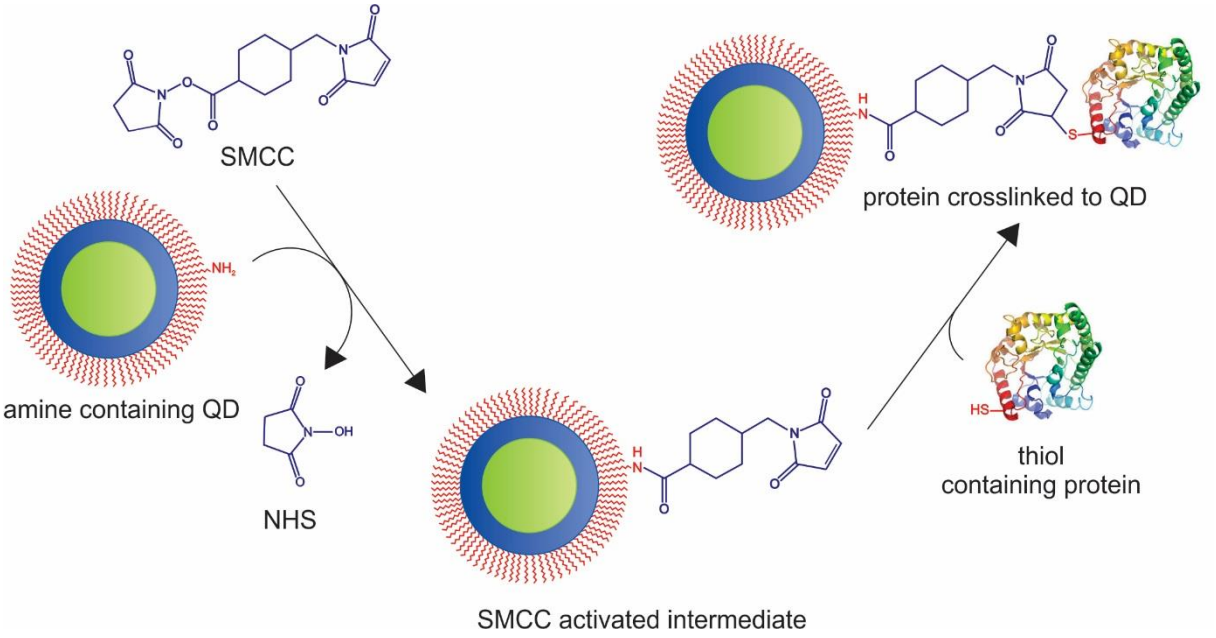

Figure 5: The two step conjugation procedure with the heterobifunctional crosslinker SMCC. In a first step SMCC reacts with an amine containing QD to form a stable amide bond. Next, the maleimide end of SMCC can be coupled with a thiol-containing molecule (e.g. protein) to yield a thioether linkage.

3.4.1. Conjugation of QDs, containing carboxyl functionality

Biggest part of the commercial available QDs are carboxyl functionalized particles, because these functional groups can be easily conjugated to free amine groups on proteins, peptides or Igs through the formation of simple amide bonds [12]. Therefore, no additional chemical modifications of the proteins before conjugation are necessary [30]. Formation of amide bonds is a simple water soluble process that usually occurs in alkaline buffered conditions which preserves structure and properties of proteins. In addition, the amide coupling does not require the use of lengthy spacers thereby preserving the hydrodynamic size of the QDs. The only increase in hydrodynamic size is directly related to the size of the functional molecule attached to the QD surface [12]. But in aqueous solutions, the carboxylate functional group displays rather low nucleophilicity and therefore it is almost unreactive with the great majority of bioconjugation agents that perform the coupling through a nucleophilic addition process [68]. Therefore, a preliminary activation of the carboxyl group is necessary.

The most well-studied and easy-to-perform method for attaching biomolecules to QDs makes use of the zero-length crosslinker carbodiimide such as EDC [70, 71]. Bioconjugation happens by direct formation of the amide bond between terminal carboxyls on the QDs and amines on the biomolecule (e.g. proteins), although the reverse configuration may work equally in some cases [29]. In general, EDC is cheap, easily obtained, and there are many protocols readily available for reference. However, single use of EDC often leads to a low yield because the reactive intermediate *o*-acylisourea tends to rapidly hydrolyze in aqueous solutions [30]. Therefore, in practice, EDC is often used in combination with water-insoluble NHS or water-soluble sulfo-NHS to form a stable active intermediate by converting it in an ester, increase solubility and increase the yield of conjugation [43, 68]. Speranskaya and co-workers compared these two binding strategies using QDs covered with poly(maleic anhydride 1-octadecene) (PMAO)-Jeffamine M1000. The efficiency of the reaction was determined by gel electrophoresis and revealed that only the combination (EDC/sulfo-NHS) protocol

resulted in a coupling of the QDs with ovalbumin[17]. But it should always be taken into account that the reaction is pH dependent and requires a large excess of EDC due to the latter's extremely rapid competing hydrolysis [43]. Some studies report a lower QY when using EDC/sulfo-NHS for bioconjugation attributed to the effect of the low pH required for this reaction. Significantly higher yields were observed for bioconjugation reactions done with glutaraldehyde and cysteine stabilized CdTe QDs which can be carried out at a higher pH [72]. The presented synthetic route has some obstacles for labeling of proteins as they contain multiple amines and carboxyls on their amino acid side chains, in addition to their N and C termini, which can result in uncontrolled heterogeneous orientation and valence on the QD and undesirable crosslinking (protein-protein, QD-protein-QD). Consequently, careful washing steps to remove the excess of chemical reactants and proteins are needed[29]. Despite, issues of crosslinking, insoluble intermediaries, competing hydrolysis, and lack of control over final orientation still remain problematic [43, 68]. Therefore, this strategy often serves as the first in series of multistep modifications of QD surface by adding other functionalities.

It is important to highlight that the pI of proteins can play a determining factor in the efficiency of a bioconjugation. While the reaction works for the conjugation of anionic proteins (BSA and myoglobin) to QD-COOH, it was not efficient for labeling of cationic proteins (cytochrome c and lysozyme). The pI of cytochrome c (~10) and lysozyme (~11) maintained the primary amines (those accessible for conjugation) in a protonated state at pH 9.2, thus inhibiting the bioconjugation. This results in an eventual hydrolysis of QD-NHS leading to the formation of the QD-COOH [73].

The QD coating also has an influence on the bioconjugation strategy. Coupling of CdSe/ZnS core-shell nanocrystals covered with bidentate surface ligands composed of DHLA or TGA was not reproducible and resulted in macroscopic aggregates when a simple EDC conjugation strategy was used. To avoid this problem introduction of PEG or a combination of DHLA and PEG can be used for the hydrophilization of QDs [74]. These limitations underscore the need for the development of new approaches for surface functionalization of QDs to improve their stability and broaden their resistance to environmental changes. Sometimes it is necessary to increase the length of the crosslinker, for example, for providing an additional spatial (conformational) freedom for attachment of biomolecules (e.g. DNA [28]) or insuring the preservation of the affinity of Ig (e.g. IgG [71]). In this case EDC can be used to first attach an amino acid, e.g. lysine or PEG-lysine, and then the obtained linker can be coupled to the biomolecule by the above mentioned procedure.

Bioconjugation can alter the optical properties of QDs. For example the absorption peak can shift (e.g. blue shift [70] or red shift [75]) after binding. The potential hypothesis can be the reduction of the QDs surface charge, polarization rate of the surrounding molecules and the Stokes shift [70]. However, there exists some controversy upon the effect of the conjugation on the QD optical properties. Some authors report an increase [8] while others report a decrease [76] or no change at all [28] in photoluminescence signal after conjugation. Consequently, a change in photoluminescence signal is often used to test the binding between a biomolecule and QD but in general the QDs should be synthesized in such a way (e.g. good passivation) to avoid any significant changes in the optical properties after conjugation with biomolecules.

QD-COOH can also function as a label for glycoproteins by performing reductive amination. Bioconjugation of the polysaccharide chains within the Fc region of an Ig has the advantage that the coupling occurs relatively far away from the antigen binding site. The carbohydrate hydroxyls can be

oxidized by using sodium periodate with formation of aldehydes which are highly reactive towards primary amines and hydrazides. The carbon-carbon bond is cleaved between adjacent hydroxyl groups. QD-COOH is not reactive towards aldehyde groups so a derivatization with a bis-hydrazide compound, e.g. adipic acid dihydrazide (ADH), is required [77]. The use of this bifunctional hydrazide (instead of hydrazine itself) provides a built-in spacer to accommodate greater steric accessibility. Hydrazide functionalities don't spontaneously react with carboxylate groups, so an activation with another compound (e.g. EDC) is necessary [73]. The drawback is that ADH, being a homobifunctional crosslinker, can introduce undesirable side reactions with formation of a closed ring structure on the QD surface. However, it is suspected that the length of the spacer arm of the crosslinker is not enough to form such ring structure. Another more likely scenario involves the cross-reaction between a derivatized QD (QD-hydrazide) with an underivatized QD (QD-COOH) with formation of a QD-QD aggregate. This can be minimized by using excessive quantities of ADH during derivatization. This is also the case for IgG which can contain numeral polysaccharide chains with some reactive aldehydes. In this case uncontrolled formation of -QD-IgG-QD- polymers occurs. Reduced reaction times, optimal temperature, and mildly acidic pH conditions may help to prevent undesirable conjugates [78].

3.4.2. Conjugation of QDs, containing amine functionality

Amine groups are the second most popular functional groups for bioconjugation after carboxyl groups. For bioconjugation of QDs-NH_2 various heterobifunctional crosslinkers are employed. Conjugation of QDs-NH_2 can happen via thiol groups of biomolecules, which is more selective and prevents crosslinking and other side reactions because only 2 amino acids, cysteine and methionine, contain thiol groups. Due to homogeneous orientation of Igs on the QD surface, this approach results in nanoprobes with a higher specificity than EDC-mediated random conjugation does. One of the most common used reagents for this purpose is the water-soluble sulfosuccinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (sulfo-SMCC) which is able to form stable amide bonds. The NHS ester end of sulfo-SMCC can react with primary amine groups. Next, the resultant QDs can form a stable thioether bond with a thiol-exposed Ig at a pH of 6.5-7.5 [79]. Despite the activated QD (QD-maleimide) being relatively stable at physiological pH, temperature is an important factor to control; as higher temperatures above room temperature can cause acceleration of the hydrolysis reactions. Hydrolysis of the maleimide moiety forms maleamic acid that is unreactive towards free thiols. Hydrolysis is also dependent on the type of chemical group next to the maleimide function. For instance, the cyclohexane ring of SMCC provides increased stability towards maleimide hydrolysis probably due to its steric effects and lack of aromatic character [73].

Disulfide bonds of Igs can also be first reduced using DTT or 2-mercaptoethylamine (2-MAE). The exposed thiols (hinge region) are sufficiently far away from the hypervariable region for QD-bioconjugation to occur [58]. The resultant active fragments of full-size Igs readily interact with maleimide-, pyridyl disulfide-, or iodacetyl-activated QDs to form a covalent bond. It has been shown that conjugation through thiol groups of reduced Igs may preserve the integrity of the Ig active site in the case of mild reduction only. Heterobifunctional NHS-maleimide crosslinker-based conjugation of all these light chain fragments to QDs strongly limits the number of functional capture molecules in a nanoprobe and their capacity to bind the target. To solve this problem, Mahmoud et al. (2011) [80] have developed an advanced procedure for partial reduction of Igs using 2-MAE or low concentrations of DTT (0.075 mM). Under these reaction conditions, the disulfide bonds between the

heavy chains are reduced, while the disulfide bonds between the heavy and light chains remain intact. This results in a high yield of functionally active 75 kDa fragments. It was also shown that 2-MAE was more selective than DTT in the reduction of disulfide bonds between the Ig heavy chains, thereby receiving a higher yield. Conjugation of these fragments by using a NHS-maleimide crosslinker (such as SMCC) resulted in a probe which was 26 times more sensitive than the QD-Ig probes prepared by the standard, non-selective reduction, method [30].

Speranskaya and co-workers conjugated PMOA-Jeffamine ED-2003 encapsulated QDs with ovalbumin by using a combination of sulfo-SMCC and SPDP. The coupling process comprised several steps. QD and protein were activated with sulfo-SMCC and SPDP, respectively; afterwards QDs were coupled with protein[17]. SPDP was used for creating thiol groups on the protein surface. Once modified with SPDP, the protein was treated with DTT to release the pyridine-2-thione leaving group and form the free thiol. The terminal -SH group on the protein then could be used to conjugate with maleimide functionalities on the QD and create a covalent conjugation [68].

An Ig can also be labeled through its polysaccharide chain with a QD-NH_2. Hereby, the carbohydrate hydroxyls can undergo oxidation (using periodate) which result in the formation of aldehydes that are highly reactive towards primary amines [73]. Aldehydes can react with amines to form Schiff base intermediates that are in equilibrium with their free forms. However, Schiff base formation is a relatively labile, reversible interaction that is readily cleaved by hydrolysis in aqueous solution. This interaction can be avoided by reductive amination. Addition of sodium borohydride or sodium cyanoborohydride to this reaction medium will result in reduction of the Schiff base intermediate and covalent bond formation, creating a secondary amine linkage between the two molecules. Cyanoborohydride is a milder reducing agent. Although cyanoborohydride does not reduce aldehydes, it is very effective at Schiff base reduction. Thus, higher yields of conjugate formation can be realized using cyanoborohydride instead of borohydride [81]. The drawback of this conjugation strategy is the degree of uncontrollability of the resulting conjugate, as undesirable protein aggregation can occur through the presence of primary amines on the biomolecules' surface. The procedure discussed above (functionalization with hydrazides) is more selective as conjugation is occurring exclusively on the polysaccharide chain [58].

Conjugation of QDs-NH_2 with other amine-containing molecules can happen with another popular homobifunctional crosslinker glutaraldehyde [82]. However, it was demonstrated by Beloglazova et al. (2013) that the use of glutaraldehyde led to a very strong non-specific interaction based on side crosslinking reactions [19]. Gluteraldehyde can form polymer-containing points of unsaturation (α,β-unsaturated gluteraldehyde polymers), which make them highly reactive towards nucleophiles (especially primary amines). The proposed reaction mechanism of conjugation using these polymer conjugates explains the stability of proteins crosslinked by glutaraldehyde. Schiff base formation alone is not able to yield stable crosslinked products without the simultaneous reduction (reductive amination). In addition, crosslinking based on gluteraldehyde polymers is difficult to reproduce and therefore the exact nature of the conjugates is hard to be determined. Storage conditions of glutaraldehyde solution used for synthesis must be also taken into consideration. Freshly opened glutaraldehyde often will not yield the same results as aged solutions [83].

Molecules containing phosphate groups, such as the 5' phosphate of oligonucleotides, can be conjugated to QDs-NH_2 by using a carbodiimide-mediated reaction (phosphoramidate formation).

The carbodiimide activates the phosphate to an intermediate phosphate ester similar to its reaction with carboxylates. In the presence of an amine, the ester reacts to form a stable phosphoramidate bond. However, to the best of our knowledge no published research has devoted this strategy to couple oligonucleotides to QDs. A possible explanation may be the potential of EDC to react with the guanosine N-1 site or with thymidine residues. Although research [84] showed that the cross-reactivity appears to be low to still maintain biological activity and hybridization efficiency.

In case that hydrophobic carboxyl containing molecules need to be coupled to QDs-NH_2, DCC in combination with NHS are often used. DCC and NHS are both water-insoluble compounds and the active ester synthesis occurs in organic solvent, and therefore the hydrolysis problems of water-soluble EDC-formed esters do not contribute to this process. A potential undesirable effect of using DCC is the spontaneous rearrangement of o-acylisourea to an inactive N-acylurea. The rate of this rearrangement dramatically increases in aprotic organic solvents, such as DMF [85]. O-acylisourea-activated carboxylates may undergo two-side reactions that form other active groups. Without presence of an amine-containing molecule, a DCC-activated carboxylate can react with another carboxyl group to form a symmetrical anhydride. In addition, a DCC-activated carboxylate may react with an amino acid to form an azlactone. Both, the anhydride and the azlactone are able to react with amines to form covalent amide linkages. However, a ring-opening reaction of azlactone can form a different product than the zero-length crosslinking result of coupling directly to an amine-containing molecule [86]. Xu et al. (2014) used the DCC/NHS protocol to activate oximated aflatoxin B_1, followed by coupling to QDs-NH_2 [47].

Targeting proteins with QDs in live cells often happens by using a biotin-SA strategy, which form quasi-irreversible complexes. For multiplexed, generic labeling of proteins on the cell surface, further targeting strategies which result in monovalent QD bioconjugates are required. In practice, this means that, if you want to create such a bioconjugate, you will often need to employ several different bioconjugation strategies. You et al. (2010) coupled PEG-coated and QDs-NH_2 with hetero-bifunctional crosslinker, 3-maleimidopropionic acid NHS ester (MPA-NHS). Next, this QD-PEG-maleimide was incubated with Tris-NTA-EG7-disulfide (EG7: hepta(ethyleneglycol) succinimidylpropionate disulfide). The resulted bioconjugate was loaded with Ni^{2+} ions, which allowed specific immobilization of His-tagged proteins. To control the degree of functionalization with Tris-NTA on the QD surface, the reaction was performed at low ionic strength, which resulted in a 1:1 functionalization. This observation suggested that electronic repulsion may steer the reaction, in contrast to the Poisson distribution. This means that once a nanoparticle is conjugated with the first Tris-NTA molecule, its highly negative electronic potential (six negative charges) efficiently shields coupling of a second Tris-NTA. Coupling at higher ionic strength yielded QDs with a substantially higher degree of functionalization. These QD-conjugates could capture His-tagged proteins *in vitro* and in live cells which allowed unbiased single-molecule tracking [87].

3.4.3. Conjugation of QDs, containing thiol functionality

Thiol functionalized QDs (QDs-SH) can be attached to sulfur containing biomolecules like amino acids such as cysteine. Disulfide bonds are covalent in nature and can be used to form zero length bonds between QDs and target biomolecules [12]. Hydrophobic QDs can be directly solubilized and functionalized based on the formation of disulfide binding of QDs with peptides, binding specifically to thiols of QDs. This procedure has been explored as an alternative to the two step solubilization

and functionalization procedure. The advantage of using peptides (e.g. phytochelatin related peptides containing an adhesive part which consists out of multiple repeats of cysteines, followed by a flexible hydrophilic linker [88]) over other compounds lies in the ability to customize various types of peptides that can act as anchoring or targeting agents [12]. When thiols are used as modification site it is important to make sure that the QD surface (especially any S-containing groups) is sufficiently protected from their reactivity [51]. As mentioned before, maleimide coupling can be used to conjugate biomolecules on the surface of QDs through a sulfo-SMCC mediated linker reaction. Sulfo-SMCC acts as spacer as well as linker molecule and increases the overall size of the QDs. The stability of this bond in cellular media is higher than the disulfide bonds as it can undergo disulfide exchange reaction in cellular media with a variety of competing thiol groups. Such a scission can be used strategically to deliver drug loaded nanoparticles/QDs to the specific target where the QDs are released with the cleavage of the thiol bonds by selective enzymes present at the cellular site [12]. The double bond of maleimides may undergo an alkylation reaction with thiol groups to form stable thioether bonds. Maleimide reactions are specific for thiols in the pH range of 6.5-7.5. At pH 7.0, the reaction of the maleimide with thiols proceeds at a rate 1000 times greater than its reaction with amines. At higher pH values, however, the reaction with amines becomes more evident. One of the carbons adjacent to the maleimide double bond undergoes nucleophilic attack by the thiolate anion to generate the addition product. When sufficient quantities of thiol groups are being alkylated, the reaction may be followed spectrophotometrically by the decrease in absorbance at 300 nm as the double bond reacts and disappears [79].

Compounds containing a disulfide group are able to participate in disulfide exchange reactions with another thiol. The disulfide exchange (also called interchange) process involves attack of the thiol at the disulfide, breaking the -S-S- bond, with subsequent formation of a new mixed disulfide comprising a portion of the original disulfide compound. A pyridyl dithiol is perhaps the most popular type of thiol-disulfide exchange functional group used in the construction of crosslinkers or modification reagents. For instance, a reagent system containing a pyridyl disulfide group, such as SPDP, is able to react with thiol groups by releasing the electron-stabilized compound, pyridine-2-thione (Figure 6). Since the leaving group does not possess a free thiol, it cannot undergo disulfide exchange with another molecule of the attacking thiol compound. Thus, only one end of the reagent has potential for becoming attached to the thiol-containing molecule [89]. Disulfide exchange reactions occur over a broad range of conditions -from acid to basic pH- and in a wide variety of buffer constituents. Most crosslinking reactions involving disulfide exchange are done under physiological conditions or those most appropriate to maintain stability of the protein or other molecule being modified. The reduction of disulfide groups to thiols in proteins using thiol-containing reductants (DTT, TCEP, 2-mercaptoethanol or 2-MAE) proceeds through the intermediate formation of a mixed disulfide. Disulfide reduction occurs over a broad pH range and in a variety of buffer environments. The reaction can be done in denaturants, chaotropic agents, detergents, and in high salt conditions [83].

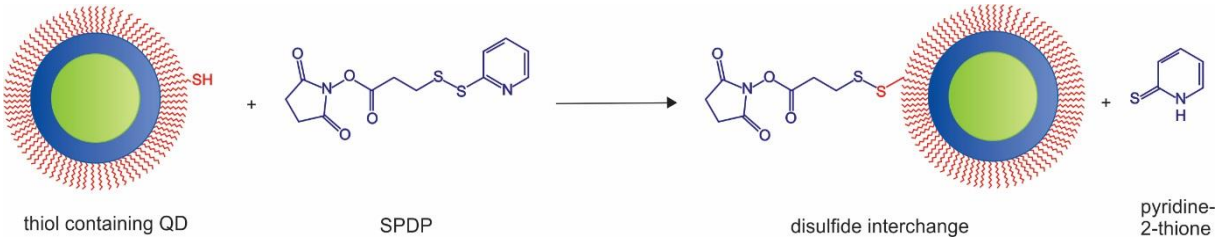

Figure 6: Thiol-disulfide exchange reaction. SPDP can react with the QD-SH, with release of pyridine-2-thione.

As already mentioned before, SPDP can also be used to incorporate thiol groups in biomolecules. Here, the NHS ester end of SPDP reacts with amine groups to form an amide linkage, while the 2-pyridyldithiol group at the other end can react with thiol residues to form a disulfide linkage. Next to SPDP, analogs are also commercially available such as a long-chain version LC-SPDP and a water-soluble, sulfo-NHS form which also contains an extended chain, called sulfo-LC-SPDP [29]. However, the disadvantage of using SPDP to create thiol on proteins is necessity of using a reducing agent to remove the pyridine-2-thione group. By this also indigenous disulfides within a protein, will be cleaved and reduced. This method therefore works well for proteins containing no thiols or no disulfides that are critical to function, but it may cause loss of activity or subunit breakdown in proteins containing essential disulfides.

One of the main hydrophilization strategies for QDs is the coating with silica. The use of silica for developing water-soluble QDs is advantageous for applications such as bioconjugation, because silica surfaces are easy to functionalize, provide an enhanced colloidal stability in polar solvents and are stable over a wide range of conditions (pH, ionic strength). In addition, silica is relatively biocompatible and protects the surface of the QDs from oxidation [6]. One of the reagents that can be used for the silica encapsulation is mercaptopropyltris(methoxy)silane (MPS), which incorporates also thiol groups on hydrophobic QDs, available for coupling. The presence of a large amount of negative charge ensures the long range repulsive interactions between nanocrystals that overwhelm their short-range van der Waals attractions. Bioconjugation of these QDs led to aggregation due to the decrease of the amount of negative charge [7]. This was also observed by Uyeda et al. (2005) who functionalized QDs with carboxyl groups. Design of the nanoparticles asks for an optimal balance between inert and active functional groups. Therefore, QDs can be embedded in a siloxane shell and functionalized with thiol and/or amine groups and phosphonate groups [8, 76] or with PEG [91] and thiol-terminated biolinkers. This will result in stable QDs, while the thiol groups serve as anchor group for bioconjugation [7, 74]. These QDs are potentially useful for a number of applications in biolabeling and imaging [90].

Besides silica-coated QDs, hydrophobic QDs can also be encapsulated with polymers. Recently, a new method was developed to conjugate DNA onto polymer-coated QDs. The polymer used to cover the QDs displays both anchoring (thiol-functionalized monomer) and water-solubilizing (zwitterion-based monomer) groups. TCEP was used to reduce the water-soluble QDs in order to regenerate free thiols (QD-SH) on the polymer. Since phosphine-based molecules have affinity towards the QD surface, TCEP can replace the original thiolated polymer. At high excess of TCEP, sufficient numbers of thiols are replaced causing the polymers to desorb from the QD surface thereby making the QDs unstable in solution. Salt concentration has also an influence on the efficiency of the reaction [28].

3.4.4. Conjugation of QDs, containing epoxide functionality

QDs can be covered with a coating that contains epoxide functionalities. They can react with different functional groups, which are present on the biomolecules. An epoxide group reacts with nucleophiles in a ring-opening process. Reaction with primary amines, thiols, or hydroxyl groups results in secondary amine, thioether, or ether bonds, respectively (Figure 7). During the coupling process, ring opening forms a β-hydroxy group on the epoxide compound. The reaction of epoxide functionalities with hydroxyls requires high pH conditions, usually in the range of pH 11-12. Amine nucleophiles react at more moderate alkaline pH values, typically needing buffer environments of at least pH 9.0. Thiol groups are the most highly reactive nucleophiles with epoxides, requiring a buffered system closer to the physiological pH range of 7.5-8.5 for efficient coupling [91]. The principal side reaction to epoxide coupling is hydrolysis. Particularly at acid pH values, the epoxide ring can hydrolyze to form adjacent hydroxyls. This diol can be oxidized with periodate to create a terminal aldehyde residue with loss of one molecule of formaldehyde. The aldehyde then can be used in reductive amination reactions. The reaction of an epoxide group with an ammonium ion generates a terminal primary amine group that also can be used for further derivatization [68].

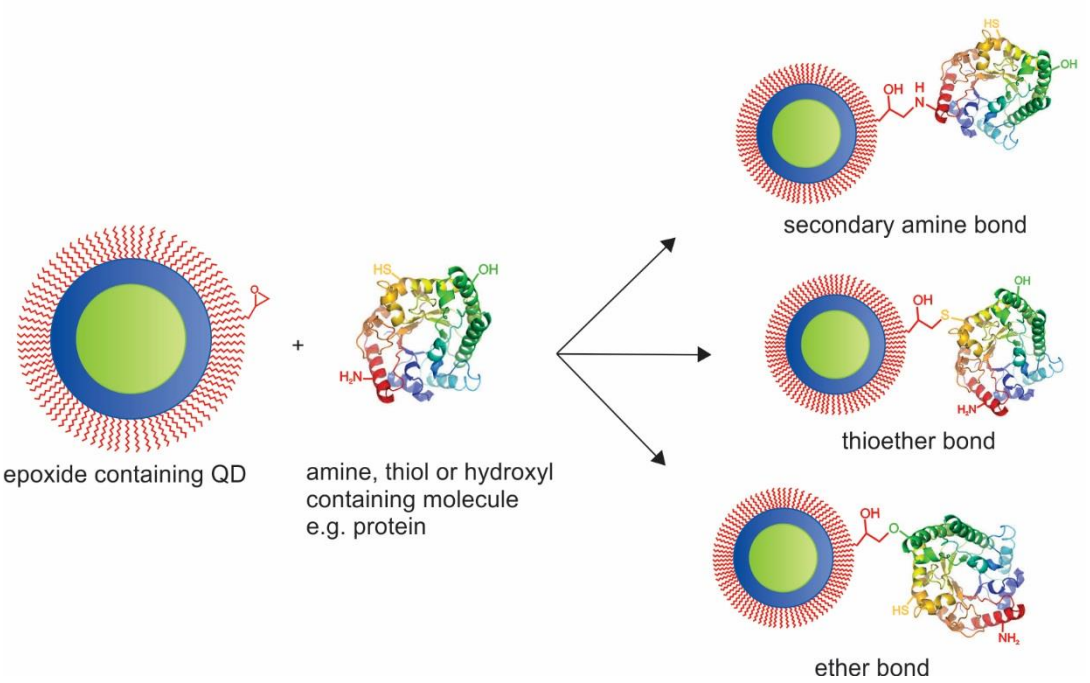

Figure 7: Epoxide containing QDs can react with amine, thiol and hydroxyl containing molecules in a ring-opening process.

However, not much is published on the promising conjugation of biomolecules with epoxide-functionalized QDs. The use of QDs coated with silica containing epoxide groups would combine the advantages of silica and epoxide groups i.e. the stability and biocompatibility that is provided by the silica layers with the ease of coupling proteins because of the reactivity of the epoxide groups. Epoxide-functionalized silicon QDs were already synthesized from alkene functionalized QDs by microemulsion synthesis [92]. Publications can be found about bioconjugation of epoxide-silica nanoparticles. For example Luzinov's group synthesized fluorescent silica nanoparticles with reactive epoxide functional groups and subsequently conjugated them with carboxy/hydroxyl groups on a fiber surface [93]. The other way around, where epoxide containing molecules are coupled to QDs-COOH is already published [94]. To our knowledge, we were the first to synthesize and use epoxide-

functionalized silica QDs for the development of a multiplex immunosorbent assay for the simultaneous detection of three mycotoxins [95].

3.4.5. Conjugation of QDs, containing hydroxyl functionality

Reliably attaching oligonucleotides to QD surface via surface carboxyl groups proved to be problematic, leading to inefficient oligonucleotide loading on the QDs and poor long-term stability. To solve this problem some researchers started to make QDs water-soluble by incorporating hydroxyl groups (e.g. QDs capped with DHLA-PEG-OH [74] or DTT [96]). Surface modification of QDs with hydroxyl groups leads to solubility properties very similar to those of QDs-COOH. In addition it has been shown that protein adsorption to hydroxylated surfaces has reduced non-specific binding [96]. Hydroxyl functionalized QDs (QDs-OH) can also be further derivatized to provide a range of functional groups like amine, or carboxylic acid for coupling to biomolecules [97].

For silica encapsulated QDs that bear hydroxyl groups on their surface the EDC strategy can be used to form an ester linkage between the carboxyl groups of the biomolecules with the hydroxylated silica surface [12]. Another method to conjugate QDs-OH to biomolecules is by use of N, N'-Carbonyldiimidazole (CDI), which is an active carbonylating agent that contains two acylimidazole leaving groups. The compound can react with a carboxylate to form an active acylimidazole group capable of coupling with amine-containing molecules. However, CDI also can react with hydroxyl groups to create a reactive intermediate. If CDI is used to activate a hydroxyl functional group, the reaction proceeds quite differently from its reaction with carboxylates. The active intermediate formed by the reaction of CDI with an hydroxyl group is an imidazolyl carbamate. Further, this reactive group, in an aqueous coupling buffer, reacts with primary amine-containing ligands via removal of imidazole groups which results in a one-carbon spacer, formation of stable carbamate linkages [68]. The coupling process is slow and occurs in alkaline conditions (pH of ± 10). Using this strategy, several reports have been published for surface modification of QDs with Igs or other biomolecules [12]. Pathak et al. (2001) prepared multicolor QD-oligonucleotide-based probes for the detection of chromosomes using common fluorescence in situ hybridization (FISH) procedures. To minimize the non-specific interaction and to increase the stability they capped their CdSe/ZnSe QDs with DDT, which incorporates hydroxyl groups. The latter were activated with CDI and coupled to 5'-aminated oligonucleotides, forming a carbamate linkage between the QD and the oligonucleotide. The QD-bioconjugates had a strong emission, photostability and could be stored for long periods [96].

Isocyanate-containing reagents can also be used to conjugate QDs-OH to biomolecules. p-maleimidophenyl isocyanate (PMPI), can be used to couple QDs-OH with thiol-containing molecules for example exposed thiol groups of Igs [98].

Igs can also be immobilized on QDs-OH by activation of the hydroxyl group with cyanogen bromide (CNBr) with formation of cyanate esters and imidocarbonates. These groups can react with primary amines on for example Igs and the amine will react mostly with the ester, yielding isourea derivates, and partially with less reactive imidocarbonate, yielding substituted imidocarbonates. The CNBr method originates from the activation and coupling of ligands to agarose supports and is subsequently used to couple Igs to hydroxyl functionalized latex particles. However, this reaction is

not widespread due to his extreme toxicity and our research also showed that silica coated QDs and liposomes loaded QDs often lose their luminescence during this reaction.

3.4.6. Conjugation of QDs, containing aldehyde functionality

A reliable and popular method for attaching Igs or other proteins to QDs involves reductive amination where amines on protein/Ig conjugate with aldehyde groups of the QDs (discussed before 3.4.1.). This reaction involves the formation of an initial Schiff base between the aldehyde and amine groups, which is then reduced to a secondary amine by a mild reducing agent. Another advantage of this method is that the functional linkers do not cross-react with other side groups present in the side chains of natural amino acids [12]. Aldehyde functionalized QDs can also be coupled with hydrazine-containing molecules (e.g. peptide modified with a 2-hydrazinonicotinoyl group) in the presence of the nucleophilic catalyst aniline in a pH range 5-8, resulting in a highly conjugated hydrazine product. Aniline-catalyzed hydrazone ligation provides a high reaction rate at pH range 5-8 and enables the efficient coupling and labeling of biomolecules at micromolar concentrations. The reactions are highly chemoselective, which allows control over the ratio of peptides ligated per QD. In the absence of aniline catalyst the hydrazone reaction is very slow. Internal UV traceable markers are often build in the hydrazine-modified molecules, which allows easy monitoring and control of the molar substitution ratios when modifying QDs or proteins [33, 49].

4. Discussion and conclusions

In general, fluorescent probes have been found widespread and are used in countless applications. QDs, in particular, have emerged as one of the most promising labels for immunoassay detection because of their photostability and size tunable photoluminescence. They can be used to develop enzyme linked-immunosorbent assays (ELISAs) [4], lateral flow immunoassays (LFIAs) [99], Western blots [59] or in FRET studies [47, 100], to name but a few. For biosensing, the greatest potential of QD-conjugates lies in multiplexing [51, 99]. QDs play also an important role in *in vitro* and *in vivo* cell imaging [34], *in vivo* drug delivery, are often used for cellular labeling (cell surface markers and intracellular markers) and have also made a lot of progress and attracted great interest in this area [48]. These applications are all possible because of the QDs versatile surface chemistry and bioconjugation. The type of bioconjugation should be selected carefully depending on the goals and application. Most of the chemistries currently used to couple QDs to biomolecules are derived from protein labeling chemistry and, as such, focus on targeting carboxyl, amine, and, to a lesser extent, thiol groups present on proteins or peptides. The presence of these functional groups means that, in many cases, bioconjugation (e.g. by using EDC [51]) can lead to crosslinking where the biological molecules are also attached to the QD in a heterogeneous manner. This problem can be partly solved/avoided by introducing multiple purification steps to obtain the desirable conjugate. Crosslinking can eventually lead to mixed avidity, loss of affinity and sensitivity. Some applications like cell trafficking require a certain ratio of biomolecules attached per QD. Control over the biomolecule ratio can be for example gained with, amongst others, aniline-catalyzed hydrazone ligation chemistry. When the distance between the QD and the biomolecule is important, e.g. in a FRET study, it is better to make use of zero-length crosslinkers or no crosslinkers at all (electrostatic interaction or epoxide-functionalized QDs). In cellular delivery, FRET and flow immunoassays compactness has additional advantages which also needs to be taken into account during the

chemistry selection. When QDs are used in cellular environments it is more likely to choose a covalent linkage instead of a non-covalent or dative one because of competition reasons [30, 50]. Another point that needs to be addressed is the orientation of the biomolecules. For example, random orientations may suffice for hybridization applications, while controlled orientations may be needed for assembly of functionalized 3D or origami structures [29]. The conjugation strategy is also dictated by the size, chemical composition (intrinsic material QD, surface ligands in case of QDs) of both QD and biomolecule. Often compromises need to be made and the most important criterion needs to be selected for conjugation.

Recent, efforts were carried out to improve QD-bioconjugation by using biorthogonal and 'click' chemistries. In this case cognate biorthogonal groups have to be introduced onto the QD and the biomolecule. For QDs this can be done during synthesis but for biomolecules it is more difficult. Peptides, DNA and lipids can be prepared with the necessary handle site but this is not possible for larger proteins such as Igs [29]. Often this is achieved by using again standard conjugation strategies like EDC/sulfo-NHS combinations. Click chemistries originally employed copper (I)-catalyzed azide-alkylene reactions, which are highly selective because they do not react with native biological functional groups [101]. The adverse effect of copper towards the luminescent properties of QDs has led to the development of copper-free bio-orthogonal approaches [102] which are likely to grow in their application. Another attempt was done for single protein tracking by producing monovalent QD bioconjugates by means of peptide surface coatings and by the demonstration of new bioconjugation schemes employing hydrazide, aldehyde, and thiol-based [87] linkages. It is important to mention that the QD-bioconjugation chemistry belongs to a larger nanoparticle bioconjugation family and that some of these chemistries are maybe interchangeable (e.g. 'click' chemistry) [43] but until now, no single chemistry is sufficient to deliver the ideal QD-bioconjugate.

Acknowledgements

The authors would like to thank the BOF Special Research Fund from Ghent University, GOA project no. 01G02213.

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