Bio-functionalised Nanoparticles for Enzyme Sensing

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Abstract

Inorganic nanoparticles and their accompanying diverse physical properties are now virtually in routine use as imaging tools in cell-biology. In addition to serving as excellent contrast agents, their size- and environment-dependent optical and magnetic properties can be harnessed to create enzyme biosensor devices of extremely high sensitivity, whilst circumventing the numerous technical limitations associated with traditional enzyme assays.

This thesis explores new applications of noble metal and semiconductor nanoparticles (quantum dots) for the detection of a range of medically-relevant enzymes. Here, water soluble colloidal gold nanoparticles and quantum dots are modified with peptides and antibodies to afford new reagents for enzyme sensing. These bio-functionalised nanoparticle probes exhibit numerous advantages over traditional enzyme sensing systems owing to their robust and size-tuneable optical properties.

These enzyme-responsive nanoparticle systems are tailored to detect the activity of protein kinase and histone acetyltransferase activity in a simple homogeneous assay format based on Förster resonance energy transfer (FRET). Furthermore, these systems can be applied to screen for putative small-molecule modulators of enzyme function. These new assays should provide the basis for the development of a variety of new enzyme detection strategies based on nanoparticle-specific optical phenomena.
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List of abbreviations

Acetyl-CoA Acetyl co-enzyme A
ADP Adenosine diphosphate
AF647 Alexa Fluor 647
APC Allophycocyanin
ATP Adenosine triphosphate
AuNP Gold nanoparticle
BODIPY Boron-dipyrromethene
BSA Bovine serum albumin
CFP Cyan fluorescent protein
COPD Chronic obstructive pulmonary disease
DCM Dichloromethane
DHLA Dihydrolipoic acid
DIEA Diisopropylethylamine
DLS Dynamic light scattering
DMF Dimethylformamide
DMSO Dimethylsulphoxide
DNA Deoxyribonucleic acid
dUTP deoxy uridine triphosphate
EDTA ethylenediaminetetraacetic acid
EPR Electron spin paramagnetic resonance
ESI-MS Electrospray ionisation mass spectrometry
FP Fluorescence polarisation
FRET Fluorescence/ Förster resonance energy transfer
GFP Green fluorescent protein
HAT Histone acetyl transferase
HBTU O-(1H-benzotriazole-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate
HDAC Histone deacetylase
HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV Human immunodeficiency virus
HOBt N-Hydroxybenzotriazole
HPLC High performance liquid chromatography
HTRF Homogeneous time resolved FRET
HTS High-throughput screening
IgG Immunoglobulin G
LSPR Localised surface plasmon resonance
MALDI Matrix-assisted laser desorption/ionisation
MBHA 4-Methylbenzhydrylamine
MPA Mercaptopropionic acid
MWCO Molecular weight cut-off
NHS N-hydroxy succinimide
NIR Near infrared
NMP N-Methyl-2-pyrrolidone
NMR Nuclear magnetic resonance
O-PAA Octylamine-modified polyacrylic acid
PAA Polyacrylic acid
PBS Phosphate buffered saline
PEG Polyethylene glycol
pY-BSA Phosphotyrosine-modified bovine serum albumin
QD Quantum dot
SAXL Streptavidin-conjugated allophycocyanin
SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERS Surface-enhanced Raman scattering
SH2 Src homology domain 2
ssDNA Single-stranded DNA
SUMO Small ubiquitin-like modifier
TAMRA Tetramethyl-6-Carboxyrhodamine
TCSPC Time-correlated single photon counting
TIS Triisopropylsilane
TMAH Tetramethylammonium pentahydrate
TOPO Triethylphosphine oxide
UV Ultraviolet
Vis Visible
YFP Yellow fluorescent protein
Chapter 1

Introduction

1.1 Introduction

Nanobiotechnology, in its broadest sense, seeks to develop novel approaches for the treatment, imaging and diagnosis of disease or to create new tools to interrogate fundamental biological processes by exploiting unique phenomena associated with nanoscale biological materials and nanoscale bioconjugates. The ability to address such important biological problems from a nanotechnology-enabled angle often by necessity requires adopting a cross-disciplinary approach, incorporating elements of biochemistry, molecular biology, medicine, chemistry, materials science, solid-state physics and spectroscopy. For example, as a good illustration of the potential power arising from such successful integration of disciplines, highly elaborate biomolecule-nanoparticle formulations can be developed which are capable of simultaneous targeted drug-delivery to and multimodal magnetic/fluorescence imaging of tumours in live mice [15]. The ability to provide such unrivalled imaging potential is afforded by the use of metallic and semiconductor nanoscale materials which posses unique and fascinating properties.

When the dimensions of many materials are reduced to the nanometer scale, new properties unseen in the bulk material often become apparent. For instance, novel electronic, optical, magnetic and mechanical properties emerge in materials on the nanometer size regime. Nanotechnology aims to integrate these unique nanoscale phenomena into functional devices. For example, the size-tuneable band-gap of semiconductor nanocrystals provides a means to create
efficient and cost-effective photovoltaic cells and light emitting diodes [16]; the high extinction coefficients and plasmonic optical properties exhibited by noble metal nanoparticles allows them to serve as powerful biological detection reagents; magnetic nanoparticles can enable ultra high-density data storage devices [17] and carbon nanotubes can be incorporated into composite materials to confer remarkable mechanical properties [18].

The ability to specifically tailor the surface chemistry of many of these nanomaterials and functionalise with a diverse range of biological molecules allows the realisation of hybrid materials, combining nanomaterial-specific properties with environment-responsive and specific biorecognition motifs. This provides new materials with powerful applications to in vitro and in vivo imaging, drug delivery, diagnostics and advanced therapeutic strategies [2]. A notable area where nanomaterials show great potential is the field of enzyme sensing and assay development.

The sophisticated chemical transformations and cellular signaling events that occur inside living cells are mediated by a host of enzymes. Enzyme dysfunction or aberrant activity is often manifested in the development of disease states and as such much attention has been centred upon the development of small molecule-modulators of enzyme activity. This requires the ability to quantitatively measure enzyme activity and is routinely carried out using fluorescence, radioisotope labeling or immunological methods [19]. Nanoparticles offer several notable advantages compared to these traditional approaches owing to their unique photophysical and optical properties. For instance, they can serve as efficacious alternatives to fluorescent dyes due to their strong resilience to photobleaching and potential to selectively tune desired emission wavelengths to match the requirements of the assay. For example, quantum dots can be tailored to emit in the far-red region of the spectrum, where biological autofluorescence - a common source of signal interference in enzyme assays - is at a minimum, [20] and nanoparticle-based approaches in general abrogate the need for potentially hazardous radiolabeled tracers. In consideration of the numerous potential benefits of using nanoparticles in enzyme-detection, this thesis aims to develop new approaches to enzyme detection.

1.1.1 Scope of the thesis

This thesis explores the use of biologically-modified semiconductor quantum dots (QDs) and gold nanoparticles (AuNPs) to devise novel approaches to enzyme sensing through utilisation of
their unique plasmonic and photoluminescent properties. These new enzyme assays are demonstrated to have persuasive advantages over currently used methods and have potential utility in high-throughput drug screening applications. The focus of this thesis is centred upon two pertinent enzyme systems implicated in the development of numerous pathological conditions such as cancer; namely protein kinases and histone acetyl transferases. Protein kinases are an ubiquitous family of enzymes which catalyze phosphate transfer from adenosine triphosphate (ATP) to the hydroxyl terminated side chains of serine, threonine and tyrosine amino acid side chains. Kinases play a prominent role in regulation of cell-signalling cell-cycle progression and are considered to be excellent targets for pharmaceutical intervention [21]. The second enzyme family interrogated using nanoparticle-based approaches are the histone acetyl transferases (HATs). These enzymes selectively modify key amino acid residues of an evolutionarily conserved family of proteins involved in condensation of the genome within the nucleus, preservation of the genome integrity and regulation of fundamental aspects of gene regulation. HATs catalyse the acetylation of conserved lysine residues found within histone proteins serving to provide post-translational markers which recruit further effectors of DNA metabolism [22]. The ability of HATs to influence the activation of cancer-potentiating genes (oncogenes) has lead to the suggestion that specific small-molecule inhibitors may have therapeutic potential [23]. Chapter 1 provides an overview of the optical properties of quantum dots and gold nanoparticles, representative examples their use in bio-sensing and current commonly used methods to detect enzyme activity. Chapter 2 describes initial efforts to devise a colourimetric gold nanoparticle-based sensing system for protein kinases in which antibody-modified gold nanoparticles are designed to undergo aggregation in a enzyme-dependent manner. Chapters 3 and 4 concern the development of an alternative energy-transfer approach based on the use of QD-peptide conjugates. As a necessary step to realise this goal, Chapter 3 details the conjugation of QDs to synthetic peptide substrates for protein kinases and further characterisation using gel electrophoresis and time resolved photoluminescence. Following from these results, Chapter 4 demonstrates the utility of these QD-peptide conjugates as surrogate protein kinase substrates that can be used in a new enzyme assay based on Förster resonance energy transfer (FRET). Further evidence suggests that this assay may have utility as a drug discovery tool using a model small molecule protein kinase inhibitor. Chapter 5 extends from this kinase assay principle to demonstrate that
it can be readily tailored to detect the activity of other enzymes, as illustrated with application to histone acetyl transferases. This suggests that the enzyme sensing strategies developed here provide a generic platform for the detection of diverse and functionally unrelated enzymes, providing a potentially valuable addition to the enzymology and drug-discovery toolbox.

1.2 Quantum Dots

1.2.1 Structure, synthesis and optical properties

Quantum dots (QDs) are crystalline semiconductor materials, typically of dimensions nanometers to tens of nanometers which exhibit optical and electronic properties governed by their size, composition and morphology. As such, they are considered to be powerful materials for the construction of high-efficiency solar cells and light emitting devices [24]. Furthermore, their robust photoluminescent properties, resistance to photobleaching, chemical stability, large effective Stokes shift and narrow tuneable emission allow them to serve as powerful alternatives to fluorescent dyes used routinely in biological imaging [25]. Figure 1.2 presents a representative high resolution TEM image of an individual quantum dot and illustrates their nanoscale dimensions and crystalline structure.

QDs typically consist of elements from periodic groups II-VI or III-V (e.g. CdSe, CdTe, InP, InAs), although more complex alloyed formulations exist [26]. They are often prepared by means of rapid injection of organometallic precursors into a high-boiling and coordinating solvent, resulting in rapid and homogeneous particle nucleation, followed by a slower growth period, yielding high-quality particles of narrow size-dispersion [27] [28]. Furthermore, a second lattice-matched inorganic shell of a higher band-gap material is often grown epitaxially upon the as-formed particle to form core-shell structures, so as to passivate non-radiative recombination sites arising from surface defects, shield the core from photooxidation and improve quantum yields [29]. Alternative methods of synthesis have been devised which greatly improve upon the scalability of QD synthesis by employing preformed molecular clusters which serve as sites for homogeneous nanocrystal nucleation. The distinct separation of nucleation and growth affords high control of nanocrystal size and polydispersity and obviates the need for hazardous or pyrophoric precursors [30].
QDs exhibit an electronic band structure intermediate between that of bulk semiconductors and atomic systems, with the band gap energy, $E_g$, determined by the size of the nanocrystal as its dimensions become of the same order as the size of the Bohr exciton radius [31]. These size-dependent electronic properties are analogous to particle-in-a-box-like quantisation of energy levels. Confinement of the electron and hole pair wavefunctions within a potential well results in the valence and conduction bands of the material no longer forming continuous energy bands, but discrete energy levels, with the band gap separation increasing as the particle size decreases (Figure 1.1). For example, in the simple case of modeling a quantum dot as a box of dimensions $l_x, l_y, l_z$, the energy levels of electrons in such a system take specific values, given by

$$E_n = \frac{\hbar^2}{8m_e} \left[ \left( \frac{n_x}{l_x} \right)^2 + \left( \frac{n_y}{l_y} \right)^2 + \left( \frac{n_z}{l_z} \right)^2 \right]$$

where the principal quantum numbers, $n_x, n_y, n_z$ take integral values (1, 2, 3...) leading to the density of electron states adopting a series of $\delta$-functions (i.e. discrete energies). In the case of idealised spherical nanocrystalline semiconductors of radius $R$, where the electron and hole wavefunctions must satisfy the boundary conditions of $\psi(r \geq R) = 0$, Brus demonstrated that in the case of CdE (E=S or Se), the energy of the lowest energy transition, $\Delta E$, can be approximated by:

$$\Delta E = E_g + \frac{\hbar^2 \pi^2}{2R^2} \left( \frac{1}{m_e} + \frac{1}{m_h} \right) - \frac{1.8e^2}{4\pi \varepsilon \varepsilon_0 R}$$

where $E_g$ is the band gap of the bulk material, $m_e$ and $m_h$ are the electron and hole effective masses, $e$ is the electron charge and $\varepsilon$ and $\varepsilon_0$ are the relative permitivities and permittivity of free space respectively [32]. The first term is the band gap of the bulk material, the second term describes quantum confinement effects and the third term accounts for electron-hole Coulombic interaction which lower the energy of the first electronic transition and becomes increasingly important in weaker confinement regimes, where the radius of the QD becomes larger than the Bohr exciton radius [31].
Figure 1.2: High resolution TEM image of commercially available CdSe/ZnS nanocrystal (605 nm emission maximum) - courtesy of Mr John Dick, Imperial College, London

Following electrical or photoexcitation and picosecond non-radiative relaxation to the lowest excited state, radiative recombination of the created electron-hole pair occurs on the nanosecond timescale and results in the emission of a photon of wavelength proportional to the bandgap energy [1]. The size-tuneable band gap provides a convenient means to selectively tailor QD
Figure 1.3: Size- and material composition-dependent tunability of semiconductor nanocrystal photoluminescent emission spectra from the visible to infra-red. Adapted from [2]

absorption and photoluminescence emission characteristics across the visible spectrum to suit the particular application (Figure 1.3), leading to appreciable utility in biological imaging and sensing applications [25]. Quantum dots have several other important advantages compared to organic fluorophores due to their strong resilience to photobleaching, allowing for long observation times on the order of several hours using standard fluorescence microscopy techniques [33]. Also, the relatively long excited state lifetime preceding electron-hole radiative recombination compared to biological fluorophores also provides a convenient means to reject interference from background autofluorescence using time-gated detection, greatly improving signal:background [34].

1.2.2 Water soluble quantum dots for biological applications

Although QDs can be prepared directly in aqueous solution (e.g. CdTe, CdSe) [35][36], high-quality and bright QDs of narrow size distribution are typically prepared in organic solvents, and their surface coating (capping agent) results in solubility restricted to non-polar organic solvents. Thus for biological applications, a range of phase-transfer procedures have been developed, including ligand-exchange with hydrophilic thiols and peptides or encapsulation withing micelles,
oligomeric phosphines, silica shells or amphiphilic polymers [37] [25][38]. Depending on the synthetic route chosen, the absorbance profile, photoluminescence quantum yield, hydrodynamic dynamic radius and colloidal stability of the QD can often be substantially altered during phase transfer procedures [37]. Furthermore, the solubilisation strategies vary in their requirements for synthetic expertise and ease of purification. The choice of the solubilisation method employed is largely determined on the intended final application. For example, whilst a polyethylene glycol-based triblock copolymer QD coating could provide enhanced colloidal stability and reduced non-specific binding in the context of in vivo imaging [39], the increased hydrodynamic radius of the complex due to the polymer layer could hinder its applicability to energy-transfer based sensing [40]. Water soluble QDs of substantially smaller hydrodynamic radius can be prepared through thiol based ligand exchange, however such approaches often suffer from substantially diminished quantum yields, poor colloidal stability in low pH or high ionic strength media and are susceptible to ligand dissociation [37]. Multidentate thiols, such as dihydrolipoic acid, on the other hand, can impart greater colloidal stability owing to a lower propensity to dissociate from the QD surface and can be further derivitised with functionalised oligoethylene glycol groups for reduced non-specific binding in protein-rich media and to provide additional chemical handles (NH₂, COOH) for further modification [41].

1.2.3 Quantum Dots and Energy Transfer

1.2.3.1 FRET background

Förster resonance energy transfer (FRET) is the distance-dependent non-radiative energy transfer between an excited state donor (D) fluorophore and ground state acceptor (A). This phenomena occurs due to dipole-dipole interactions between fluorophores with overlapping excitation and emission spectra provided that the fluorophores are within sufficient proximity of each other and typically takes place on the 1-10 nm distance regime [42]. FRET typically results in diminished donor emission intensity, reduced excited state lifetime and sensitised acceptor emission. Nonetheless, energy transfer does not require the obligatory use of an inherently fluorescent acceptor as other non-fluorescent quenching agents can also be used [43]. The strong distance dependence on the efficiency of the FRET process, following a 6th power dependence on D-A separation r, is commonly used tool to measure distances between fluorophores and can be applied
to address a wide range of questions in structural biology, macromolecule interaction analysis and biological imaging from the ensemble down to single molecule level [44]. Furthermore, this strong distance dependence also allows the FRET to have great potential for homogeneous sensing assays and diagnostics as FRET acceptors free in solution do not contribute to the measured signal [45]. The distance dependence on the rate of energy transfer, \( k_T \), from a donor to an acceptor is given by:

\[
k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6
\]

(1.1)

Where \( \tau_D \) is the lifetime of the donor in the absence of acceptor, \( R_0 \) is the distance at which 50\% FRET efficiency occurs and \( r \) is the interfluorophore distance separation. \( R_0 \) takes a characteristic value for a given pair of fluorophores and can be calculated using:

\[
R_0 = \frac{9000(ln10)\kappa^2Q_D}{128\pi^5Nn^4} \int_0^\infty F_D(\lambda)\varepsilon_A(\lambda)\lambda^4 d\lambda
\]

(1.2)

where \( Q_D \) is the quantum yield of the donor, \( N \) is Avogadro’s constant, \( n \) is the refractive index of the medium and \( \kappa^2 \) describes the relative orientation of the donor and acceptor transition dipoles. The integral term describes the degree of overlap between the donor fluorescence and acceptor absorption with respect to wavelength. The overall FRET efficiency, \( E \), (i.e. the ratio of photons emitted by the acceptor to those absorbed by the donor) is given by:

\[
E = \frac{k_T(r)}{\tau^{-1}_D + k_T(r)} = \frac{R_0^6}{R_0^6 + r^6}
\]

(1.3)

Experimentally, FRET efficiency can be calculated by measuring the steady-state fluorescence intensity or excited state lifetime of the donor in the presence and absence of acceptor:

\[
E = 1 - \frac{F_{DA}}{F_D}
\]

(1.4)

\[
E = 1 - \frac{\tau_{DA}}{\tau_D}
\]

(1.5)

Various combinations of fluorescent organic dyes, proteins, lanthanide complexes, polymers
and metallic nanoparticles have been utilised in FRET-based biosensing\[46\] [47] [48][49][50]. Traditional FRET sensing suffers from several important drawbacks; for instance sensitivity of some fluorophores to photobleaching under sustained excitation, susceptibility to interference from background autofluorescence, inadvertent direct excitation of acceptor fluorophores at the donor excitation wavelength causing signal contamination and limited interfluorophore Förster distances resulting in limited energy - thus FRET is not necessarily suitable tool to measure interactions between high molecular weight biological macromolecules. [44][25].

1.2.4 Quantum dots as FRET donors

Quantum dots possess several properties that allow them to serve as particularly attractive alternatives to organic dye and protein fluorophores in FRET-based sensing. For example, the colloidal nature of QDs leads to them having a large effective surface area and provides the ability to array multiple FRET acceptor molecules around a single QD donor. In addition, as FRET efficiency is largely governed by the spectral overlap of the donor emission and acceptor excitation spectra, the QD emission can, in theory, be tailored to be complementary to any acceptor of choice by tuning the size and composition of the nanocrystal. Experiments using conventional organic FRET pairs are also complicated by the potential for non-FRET based acceptor emission due to direct excitation at the donor wavelength and often require deconvolution of composite emission spectra. This is largely a reflection of the combination of the small Stokes shift and broad excitation spectra of many organic dyes [51]. The ability to excite QDs at essentially any wavelength below the absorption onset provides a convenient means to avoid such direct excitation simply by choosing excitation windows that correspond to acceptor absorption minima [41].

An important and potentially complex consideration is whether the QD-dye FRET process is commensurate with that associated with conventional dye-dye pairs and obeys Förster formalism. It is possible to systematically study the effect of QD-dye distance dependence of FRET by using peptides containing a (YEHK)$_n$ peptide repeat motif. This motif behaves like a rigid rod-like spacer of length depending on the value of $n$. Stoichiometric conjugates of QDs and dye-labeled (YEHK)$_n$ peptides of different length exhibit an $r^6$ dependence of $E$, consistent with classical FRET formalism [52]. In the case of the use of Au nanoclusters as FRET accept-
tors which quench fluorescent dye and QD emission, systematic variation of QD-Au separation reveals an $r^4$ dependence on $E$, allowing energy transfer to occur over distances beyond those expected for a typical Förster-like mechanism [52]. In addition, modeling electronic couplings between QDs and other organic fluorophores based on time-dependent density functional theory provides results consistent with dipole approximation of FRET [53]. Finally, $E$ is dependent on the relative orientations of the transition dipoles of the acceptor and donor with respect to each other. This is given by the orientation factor, $\kappa^2$ (equation 1.2). Provided that the donor and acceptor orientations randomise by rotational diffusion prior to energy transfer, $\kappa^2$ is assumed to be $2/3$. Room temperature polarisation microscopy has shown the transition dipoles of CdSe QDs to be 2D degenerate, suggesting a degree of dipole randomisation [54]. In addition, given the relatively large size of QDs and the inability to control the precise orientation of acceptors on the surface allows the relative orientations of QD donor and dye acceptor to be on average random, thus in an ensemble of QD-dye acceptors, $\kappa^2$ can be assumed to be $2/3$ [55].

1.2.5 Quantum dots in FRET biosensing

DNA sequencing, detection of hybridisation of complementary oligonucleotides and enzymatic processing can be routinely analysed using a multitude of fluorometric approaches based on energy transfer [56]. QDs have been tailored to detect specific DNA sequences by means of energy transfer to proximal gold nanoparticle or dye quencher groups. For example, homogeneous binding assays have been developed where a target DNA sequence cross-links two distinct oligonucleotides - one labeled with a fluorescent dye and the other with a terminal biotin - by sequence-specific base pairing (Figure 1.4, D) The biotin group can further bind to streptavidin-coated QDs to form a complex with an associated FRET signature depending on the overall dye:QD ratio. This signature can be characterised by ensemble time-resolved fluorescence or detection of dye/QD coincident photon bursts in single diffusing QD-DNA assemblies by confocal microscopy [6]. Numerous systems analogous to molecular beacons have also been developed, whereby target DNA sequence hybridisation increases the distance at which a monovalent gold cluster/dye quenching agent, tethered to the QD by a ssDNA linker, is separated from the QD surface resulting in enhanced QD emission [57]. Hybridisation events with chromosomal DNA can also be imaged in fixed cells using DNA-modified hydroxy terminated QDs in a procedure
analagous to fluorescence in situ hybridisation (FISH), with such QD probes showing low levels of non-specific binding. Finally, elaborate DNA transformations such as telomerisation and replication can also be detected via QD energy transfer by virtue of enzymatically-incorporated fluorescent deoxynucleotide triphosphates analogues [58]. Here, QDs modified with a ssDNA oligonucleotide primer serve as a template substrate for telomerase - in short, a critical ribonucleoprotein complex that prevents the aberrant loss of genetic information accumulated over many cycles of cell-division and DNA replication. Texas-red labeled dUTPs are enzymatically added to the terminus of the ssDNA template, resulting in QD-dye energy transfer and allowing the observation of telomerase dynamics.

Nucleic acids can also be engineered by systematic in vitro evolution approaches to selectively recognise targets other than complementary nucleotide sequences, such as peptides, proteins and other small molecules [59]. Aptamers represent a cost-effective alternative to antibody-based sensing as they are amenable to chemical synthesis and chemical modification and are stable under a broad range of conditions [60]. The QD photoluminescence quenching properties exhibited by gold nanoparticles have been used in a multiplexed sensing assay for the parallel detection of cocaine and adenosine. Here, gold nanoparticles and different QDs with distinct emission maxima are cross-linked by means of ssDNA aptamers with specificity for either adenosine or cocaine [49, 61]. The AuNP-mediated cross-linking allows a dual mechanism of analyte detection based on both QD-specific photoluminescence and colourimetric readout indicative of the dispersion-state of AuNPs due to interparticle-plasmon coupling. The cross-linked nanoparticle assemblies exhibit quenched photoluminescence owing to the AuNP-QD proximity, however, addition of down to c.a. 120 µM concentrations of the adenosine/cocaine targets results in a structural switch in the respective aptamers, leading to nanoparticle dispersion and recovery of photoluminescence.

Distinct from nucleic-acid based systems, several assays have been developed which can measure the activity of hydrolytic enzymes, specifically proteolytic activity (Figure 1.4, A). Numerous assay configurations have been reported, which differ slightly in details of bioconjugation methods, but in essence employ a QD conjugated to a number of FRET acceptors via an intermediary protease-degradable peptide substrate. In response to proteolytic activity, the FRET acceptor is liberated from the vicinity of the QD, resulting in dequenching of QD photolumi-
nescence [62][63][64]. Through judicious selection of QD-fluorophore donor-acceptor pairs, it is possible to realise homogeneous reactions where the activity of multiple protease enzymes can be assayed simultaneously (i.e. multiplexing) [3][65]. As opposed to using QDs as energy transfer donors in protease sensing, they can also serve as efficacious acceptors in bioluminescence energy transfer. For example, in a simple approach without need for elaborate derivitization of the nanoparticle’s surface, QDs can be conjugated to the bioluminescent protein luciferase through an intermediary protease substrate [65]. Oxidation of the luciferase substrate coelenterazine results in QD photoluminescence in the absence of external illumination due to energy transfer. Proteolytic cleavage of the intermediary peptide, however, liberates the luciferase from the QD surface and thus prevents energy transfer from taking place.

It is also possible to extend QD-FRET to the detection of unrelated bacterial enzymes such as β−lactamases - an important family of enzymes involved in cell-wall processing which often confer resistance to various antibiotics [4]. Here, a Cy5-labeled, biotinylated derivative of a β−lactamase substrate quenches the emission of streptavidin-coated QDs. Energy transfer is prevented upon enzymatic cleavage of the substrate and restores QD-specific photoluminescence (Figure 1.4, B). Whilst the assay design is simple, the approach is somewhat limited by the need to synthesize the substrate analogue and the fact that the structure of the substrate, namely the intermediary linker between the hydrolyzable component and the biotin group of the substrate, was shown to have a significant influence on enzyme accessibility.

Other biocatalytic transformations can be detected using QD-specific electrical properties. For example, enzyme-dependent tyrosine oxidation can be detected using peptide-conjugated CdSe/ZnS QDs. Tyrosinase-generated quinone derivatives of tyrosine result in quenching of QD photoluminescence due to electron transfer which can be subsequently recovered upon proteolytic cleavage of the peptide [66][67].

1.2.6 Limitations of QD-FRET

Despite the numerous benefits of the use of QDs in FRET-based biosensing as evidenced by the diverse range of their applications which have been realised in a relatively short space of time, they are still limited by several important caveats. For example, commercially available water soluble QDs are normally derivitized with proprietary amphiphilic polymer or phospholipid
Figure 1.4: Examples of quantum dot-based FRET biosensing assays ([3][4][5][6]). A) Protease detection; B) β-lactamase detection using a biotinylated fluorescent substrate analogue; C) Small-molecule detection (maltose) based on displacement of a quencher analogue; D) Detection of DNA hybridisation through formation of a DNA-QD nanassembly.
coatings which contribute to the overall hydrodynamic radius, hence the distance of closest approach of the D-A pair will be limited and also the maximum attainable FRET efficiency. In addition, an additional layer of streptavidin to the QD surface for further derivitization with biotinylated molecules further contributes to the overall D-A separation and it may be difficult to ascertain the total number of available biotin binding sites per QD. Single molecule imaging and time-resolved FRET studies are limited by the inherent intermittent nature of core/shell structure QD emission (i.e. blinking). Methods to eventually circumvent this issue may include the use of QDs of more elaborate graded shell composition, such as CdZnSe/ZnSe, which exhibit continuous non-blinking emission [68]. The utility of QDs as effective FRET acceptors has been considered to be limited owing to their long exciton lifetime and broad excitation. Nonetheless, under certain circumstances, QDs can serve as energy acceptors, particularly in the case of the use of lanthanide-based donors which exhibit long luminescence lifetimes on the ms timescale. Such systems exhibit exceptionally large Förster radii providing new opportunities for long-range FRET biosensing [69]. Finally, robust and reproducible methods of preparing and purifying stable QD-biomolecule conjugates of well-defined structure and stoichiometry remain as ongoing technical challenges [70].

1.3 Gold nanoparticles

1.3.1 Synthesis, surface chemistry and biological modification

The optical properties of gold nanoparticles (AuNPs) have been used fortuitously as glass-staining additives since ancient times [71]. Monolayer protected AuNPs are now routinely prepared by a broad variety of colloidal routes in both organic and aqueous solvents, providing the ability to readily functionalise with both hydrophobic and hydrophilic surface ligands, but generally involve reduction of gold salt precursors in the presence of additional stabilising agents to resist particle aggregation. Thiols, amines, phosphines and a range of polymers or dendrimers can serve as appropriate stabilisers and are often amenable to further chemical derivitization [71]. Due to solvent compatibility, biological applications often employ water-soluble AuNPs prepared by the reduction of HAuCl₄ by sodium citrate or reduction of preformed gold nuclei 'seeds' [72]. This route provides approximately spherical particles on the 10 - 100 nm size regime.
with reasonably narrow size distributions. Surface-bound citrate molecules provide electrostatic stabilisation of the colloid, however, the labile nature of the Au-citrate interaction often necessitates replacement with higher-affinity stabilising ligands to afford greater resilience towards particle flocculation. This can be achieved through ligand-exchange reactions with hydrophilic thiols (e.g. thioalkyl oligoethylene glycols) and phosphines to render the particles less sensitive to electrolyte-induced aggregation and non-specific protein binding [73][74]. Various analytical techniques have been utilised to investigate the rich chemistry of interfacial ligand exchange reactions, including NMR, electron spin paramagnetic resonance (EPR), surface enhanced Raman scattering (SERS) and fluorescence quenching, providing insight into the structural and mechanistic details of the process with occasionally conflicting models. Overall, interfacial ligand exchange has been shown to be dependent on the structures of the incoming and displaced ligands and the size of the nanoparticle [75][76][77][78]. Nonetheless, ligand exchange approaches have provided a convenient method to functionalise AuNPs with numerous biomolecules such as DNA, peptides and proteins owing to the ease of incorporation of thiols into these molecules through well-established synthetic procedures and site-directed mutagenesis [79][80][81]. In addition to thiol/Au-driven self-assembly of AuNP-biomolecule composites, site-selective ligation reactions, such as the azide/alkyne cycloadditions can provide an alternative means of bioconjugate formation and facilitates selective biomolecule orientation on the nanoparticle surface and assists in preserving protein function relative to less selective covalent coupling methods (e.g. carbodiimide coupling) [82, 83].

Biofunctional gold nanoparticles have been proposed to serve as useful reagents in nanomedicine and have been demonstrated to serve as contrast agents for cellular imaging, drug delivery platforms, gene transfection devices and tools for targeted thermal ablation of tumors [84]. One of the most useful properties of gold nanoparticles is their intense and environment-sensitive optical absorption which allows them to provide the basis of operation for a wide range of novel biological detection probes.

1.3.2 Localised surface plasmon resonance

Well-dispersed noble metal nanoparticles, such as silver and gold, exhibit intense optical extinction spectra owing to localised surface plasmon resonance (LSPR). In contrast to semiconductor
nano crystals where quantum confinement effects govern the optical absorption and photoluminescence emission characteristics, the LSPR exhibited by gold nanoparticles (> c.a. 3 nm in diameter) can largely be described using a classical picture of electrons. LSPR arises due to coherent, collective dipolar oscillations of conduction band electrons in response to excitation with visible light (Figure 1.5) and results in an intense absorption band, known as the surface plasmon absorption band (Figure 1.6, A). All metals possess this characteristic, however noble metals (Au, Ag) and copper have particular practical utility due to their resistance to oxide formation and chemical stability and the fact their LSPR absorption predominantly occurs in the visible region of the electromagnetic spectrum [7]. The basis of the surface plasmon resonance band and the associated intense absorption properties of colloidal gold was first rationalised by Mie’s solutions to Maxwell’s equations for spherical particles of radius, $R$, significantly smaller than the wavelength of incident light ($R \ll \lambda$) and provides a relationship between the extinction cross-section, $\sigma_{ex}$ (i.e. absorption + scattering) as a summation over all electric and magnetic oscillations, given by;

$$\sigma_{ex} = 24\pi^2 R^3 \varepsilon_m^{2/3} \frac{\varepsilon_2}{\lambda} \frac{1}{[\varepsilon_1 + 2\varepsilon_m]^2 + \varepsilon_2^2}$$

Where $\lambda$ is the wavelength of light, $\varepsilon_m$ is the dielectric constant of the surrounding medium and $\varepsilon_1$ and $\varepsilon_2$ are the real and imaginary components of the dielectric constant of the metal ($\varepsilon = \varepsilon_1 + i\varepsilon_2$) [85]. The position, intensity and shape of the plasmon absorption band is dependent on a number of factors, such as nanoparticle composition, morphology (e.g. nanorods), anisotropy, size and size distribution due to size-dependent dielectric properties of the metal (intrinsic size effects). In the case of larger particles, higher-order multipolar oscillations of the conduction band electrons are also possible which can influence the plasmon band position and width (extrinsic size effects) [86, 87][71]. Furthermore, the modification of the nanoparticle local dielectric environment and refractive index due to solvent and/or surface ligand coating can also influence the plasmon absorption [86]. Interparticle proximity is another important factor which can profoundly modify the surface plasmon behaviour due to the interparticle plasmon coupling. This phenomena, associated with nanoparticle aggregates is manifested as a marked red-shift and broadening of the plasmon absorption band and typically accompanied with a
Figure 1.5: Plasmon oscillation of a metal nanosphere in response to an incident electric field [7].

red to purple/blue colour change of the particle solution, with the overall aggregate size and interparticle spacing influencing the optical properties (Figure 1.6 B) [87].

1.3.3 Colourimetric LSPR biosensing

The ability to synthesise gold nanoparticles in aqueous solvent, their rich surface chemistry and amenability to bioconjugation has enabled the use of their aggregation-dependent optical properties in numerous colourimetric biosensing systems to detect small molecules, proteins and biocatalytic transformations [88]. A paradigm example of gold nanoparticle-based biosensing is represented by the DNA-hybridisation assays of Mirkin, where thiol-DNA modified AuNPs are cross-linked by means of a complementary ssDNA target sequence [89]. This principle has since been extended to detect the activity of a range of DNA processing enzymes, such as sequence-specific endonucleases, ligases and methyltransferases, which can augment the structure of DNA-crosslinked nanoparticle assemblies [90][91][92]. Other elaborate colourimetric sensing systems based on DNA-conjugated AuNPs have been devised which exploit conformational changes induced upon binding of DNA aptamers to diverse target molecules, such as heavy metal cations, alkaloids, proteins and cancerous cells (Figure 1.7, E) [49][60][93][94]. Heterostructured gold
Figure 1.6: (A) Normalised optical absorbance of gold nanoparticles, as determined by UV-Vis spectroscopy, of different dimensions [8] and (B) aggregation-dependent plasmon band broadening [9].
	nanostructures, such as gold SiO$_2$/Au nanoshells, provide an alternative means to tune the plasmon absorption band to wavelengths significantly longer than those exhibited by typical solid-gold nanoparticles to the NIR and beyond [95]. This allows the realisation of colourimetric immunoassays based on aggregation-dependent plasmon red-shifting which operate in spectral windows where biological matrices, such as plasma and blood, are particularly transmissive. For example, antibody-coated nanoshells can be induced to form higher-order aggregates upon binding to a multivalent target antigen, resulting in a shift in absorption spectrum which can be used to detect sub-nanogram quantities of target analyte in <10 min (Figure 1.7, B) [10].

In addition to the novel properties of DNA-based hybrid nanostructures, the robust optical properties of gold nanoparticles allow them to serve as persuasive alternatives to fluorogenic or radioisotope labeled substrates in enzyme activity assays, owing to their insensitivity to photobleaching and relative safety. For example, a number of protease detection strategies have been reported which rely of proteolytic degradation of substrate peptides which can affect the aggregation of gold nanoparticles. For example, a protease substrate, containing two cysteine residues positioned to flank the hydrolysis site, can cross-link citrate-stabilised AuNPs through the divalent thiol-Au interaction, leading to particle aggregation and an associated red-shift and broadening in plasmon absorption. Proteolytic cleavage, however, separates the two cysteine
residues and prevents particle agglomeration [96]. A real-time protease assay has also been
developed based on pi-pi stacking driven association of peptide-modified AuNPs by means of
appending Fmoc groups, with proteolytic cleavage of the peptides resulting in dispersion of the
nanoparticles upon loss of the Fmoc interaction (Figure 1.7, A) [9].

Several colourimetric AuNP sensing approaches have been implemented to detect enzyme-
mediated phosphorylation by protein kinases (see following section). For instance, the substan-
tial changes in peptide charge upon phosphorylation can be exploited to either cause agglom-
eration or dispersion of AuNPs upon adsorption to the nanoparticle surface. For instance, a
non-phosphorylated substrate peptide with cationic character can elicit aggregation of anionic
AuNPs due to charge screening, whereas the anionic phosphorylated equivalent lacks this ca-
pability (Figure 1.7, C) [11, 97]. It is of note that this principle can be readily extended to
detect the antagonistic enzyme modification of phosphatase catalyzed dephosphorylation [98].
Whilst these approaches are simple, and do not require sophisticated nanoparticle derivitisation
procedures, the fact that the AuNPs in such assays are stabilised with a labile citrate coating
renders them susceptible to electrolyte-induced flocculation and/or non-specific binding to
biomolecules [99]. An alternative approach to electrostatic interaction-based detection of kinase
activity is the use of high affinity biospecific interactions. For instance, using a γ-biotin deriv-
avative of ATP, AuNPs coated with kinase substrate peptides can be enzymatically biotinylated and
subsequently bind to streptavidin-coated AuNPs, providing a convenient colourimetric response
which can be applied to screen potential kinase inhibitors (Figure 1.7, D) [13].

Colourimetric biosensing assays using AuNPs in common with all assays, are subject to sev-
eral limitations. For instance, gold-based biosensing often requires substantial engineering of
the nanoparticle surface, possibly leading to precarious colloidal stability in biological matrices
thereby limiting reagent shelf-life [100, 80]. In addition, the propensity of AuNPs to undergo
complex ligand exchange reactions with thiols and other biological molecules (e.g. DTT, re-
duced glutathione) may cause premature dissociation of biodetection motifs immobilised on the
nanoparticle surface [84]. Finally, the need for scalable, robust synthesis methods and char-
acterisation techniques of biologically-modified gold nanoparticles presents a current bottleneck
to the realisation of their widespread application in biology [101].
Figure 1.7: Representative examples of colourimetric gold nanoparticle biosensing. A) Proteolytic dispersion of aggregated nanoparticles; B) Antigen-mediated immunoaggregation; C) Electrostatic aggregation of anionic nanoparticles by cationic peptides. Alternatively, the increased negative charge of the peptide upon phosphorylation prevents aggregation; D) Kinase catalysed biotinylation of peptide-modified gold nanoparticles. Exposure to streptavidin-coated gold nanoparticles leads to interparticle crosslinking and plasmon broadening; E) Aptazyme-based detection of heavy-metal cations. The presence of the target cation results in self-cleavage of the aptazyme, preventing the cross-linking of nanoparticles derivitised with complementary oligonucleotides [9][10][11][12][13].
1.4 Protein kinases

1.4.1 Background

The cell’s ability to modify amino side chains through enzymatic post-translation modification serves to greatly enhance the diversity of proteins by introducing new chemical functionalities, distinct from the those of the standard repertoire of proteogenic amino acids, and provides new potential layers of regulation [102]. Amongst the wide range of post-translational modifications which exist, protein kinase mediated reversible phosphorylation of serine/threonine/tyrosine residues is one of the most widely employed regulatory device found in eukaryotic cells (Figure 1.8). The protein kinases are one of the largest families of proteins encoded by the human genome with more than 500 identified to date [21]. Protein phosphorylation plays a pivotal role in the integration of extracellular signals with intracellular activities and is responsible for the regulation of fundamental cellular processes, including stem-cell differentiation, embryogenesis, cell-cycle control and gene expression [103]. In consideration of the central role that these enzymes play in cell biology, the dysfunction or deregulation of protein kinase activity is often manifested in the development of numerous disease states including chronic inflammation, neurodegeneration, osteogenic disorders, cardiovascular disease and cancer [104]. This is a reflection of the fact that each of the classically defined ‘hallmarks of cancer’ (i.e. evasion of apoptosis, unlimited replicative potential, self-sufficiency from growth factors, insensitivity to anti-proliferative cues, sustained angiogenesis, cell invasion and metastasis) is intimately linked to the aberrant action or inaction of protein kinases in the cell’s regulatory circuitry [105].

In light of the central role that these enzymes play in the development of numerous diseases, protein kinases are considered to be attractive targets for pharmaceutical intervention and second only to G-protein coupled receptors in terms of significance as a drug targets [106]. As such, there is a strong impetus to develop assays and high-throughput screening procedures to provide quantitative information on kinase activity for drug discovery and a myriad of biochemical in vivo and in vitro assay technologies are in use. A host of diverse solid-phase and solution-based protein kinase assays have been developed. Thus it is necessary to provide a brief discussion of current screening approaches available and their advantages and limitations, paying particular attention to state-of-the-art techniques currently used in industrial drug discovery programmes.
1.4.2 Fluorescence polarization anisotropy and time-resolved energy transfer

Radioisotope-based methods to measure kinase activity typically employ radiolabeled $\gamma^{\text{-32P}}$-ATP as a cosubstrate, with the radiolabeled phosphate becoming incorporated into the kinase’s peptide/protein substrate followed by separation of the labeled reaction product and detection by scintillation counting or autoradiography. This method is simple, sensitive, rapid and generic, however the use of expensive and potentially hazardous isotope tracers and the non-homogeneous format (i.e. necessity for product separation from excess $\gamma^{\text{-32P}}$-ATP) prevents this approach from being broadly applicable to high-throughput screening [107][108]. Fluorescence-based detection techniques have therefore superseded radioisotope labeling owing to the combined benefits of sensitivity and amenability to miniaturisation and automated high-throughput screening.

Two of the most widely used methods to screen kinase activity are fluorescence polarization anisotropy (FP) and time resolved FRET (HTRF), owing to their homogeneous nature (i.e. no washing steps) and simplicity in operation. Fluorescence polarization makes use of the differences in rotational diffusion between macromolecules of different sizes [109]. For instance, a peptide will rotate in solution much more rapidly than a typical antibody. For example, as applied to kinase activity, a fluorescently labelled substrate peptide is free to rotate relatively rapidly in solution. Upon enzymatic phosphorylation of the peptide and subsequent binding of a phospho-specific antibody or chelating nanoparticle, the rotational diffusion of the peptide
is substantially modified owing to the increased effective mass of the complex. The signal is generated upon illumination with vertically polarised light and detecting the emitted light intensity parallel ($I_\parallel$) and perpendicular ($I_\perp$) to the plane of excitation. This generates a dimensionless anisotropy ratio $r$ [42]:

$$r = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp}$$

The ratiometric nature of the FP signal allows the measurement to be less prone to well-to-well variations due to the presence of coloured samples and is independent of the total fluorophore concentration, however the approach is sensitive to interference due to scattering and autofluorescence, although these effects can be reduced through the use of fluorophores with red-shifted emission profiles [20]. Furthermore, FP requires the use of antibodies and is restricted to low molecular weight peptide substrates. Finally, the FP assay is a loss-of-signal format which is inherently limited by the fact that a relatively high enzyme turnover is required prior to producing a statistically significant signal above the background [107].

Homogeneous time-resolved FRET (HTRF) is one of the most powerful techniques available to biomolecular screening owing to its greatly diminished sensitivity to background autofluorescence. HTRF makes use of the unique luminescent properties of certain rare lanthanides such as europium (Eu$^{3+}$) and terbium (Tb$^{3+}$) [14]. These metals exhibit exceptionally long luminescence decay times on the order of milliseconds when encapsulated within a suitable chelating group. The chelate serves to relay excitation energy to the lanthanide due to very low absorption coefficients of the lanthanides in isolation, enhance the quantum yield of the lanthanide and provide functional groups for bioconjugation [110]. Typically, an anti-phosphoaminoacid antibody is conjugated to the lanthanide chelate (cryptate) which can bind to a biotinylated phosphopeptide. The biotin, in turn, serves to bind to a streptavidin-conjugate of the fluorescent protein allophycocyanin (APC), known as SAXL. Assembly of the complex results in energy transfer from the cryptate to APC. By exciting the cryptate with a brief pulse of illumination and detecting cryptate and APC fluorescence following a time-delay, residual background autofluorescence can be effectively eliminated from the measured signal owing to its tendency to decay on the order of nanoseconds (Figure 1.9) [14].
Figure 1.9: Principle of time-gated HTRF measurement [14]. The sample is briefly illuminated with an excitation pulse at 337 nm, followed by a time delay to allow for the decay of autofluorescence originating from biological matrices and test compounds etc. Energy transfer in the Eu-cryptate and SAXL complex is subsequently detected by measuring the emission intensities at 665 nm and 620 nm.

HTRF represents one of the most versatile and robust assay technologies available. The assay is somewhat limited by the need for costly cryptate-labeled phospho-specific antibodies and preparation of high-molecular weight APC conjugates.

1.4.3 Fluorescent synthetic peptides and genetically-encoded fluorescent reporters

FRET assays for kinases can also be used in antibody-free context, using substrate peptides labeled with both a FRET donor and acceptor. Phosphorylation of the peptide alters its sensitivity to proteolytic cleavage which results in the separation of the donor and acceptor and changes in FRET efficiency [111]. This is a convenient assay amenable to miniaturisation but requires care in substrate design due to limited FRET for higher molecular weight peptides and may be susceptible to off-target effects in the search for kinase inhibitors due to the coupled-enzyme detection format. To circumvent the limitations imposed by the sensitive distance-dependence
Table 1.1: Comparison of currently available protein kinase assay technologies

<table>
<thead>
<tr>
<th>Technology</th>
<th>Assay principle</th>
<th>Advantages</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>FRET</td>
<td>Differential protease sensitivity of D-A dye-labeled protein kinase substrate peptide</td>
<td>Homogeneous, high-throughput, antibody-free</td>
<td>Different substrate design, coiled enzyme assay</td>
</tr>
<tr>
<td>HTRF</td>
<td>Energy-transfer between Eu(^{3+})/Tb(^{2+})-labeled phosphopeptide antibody and dye-labeled substrate peptide: allophycocyanin-conjugated streptavidin</td>
<td>Low background signal</td>
<td>Antibody-dependent, limited choice of D/A pairs, stability of chelate</td>
</tr>
<tr>
<td>AlphaScreen</td>
<td>Photogenerated singlet oxygen proximity-dependent luminescence. Photosemifluorescent donor and singlet oxygen-sensitive chemiluminescent beads bind to each other in response to phosphorylation of a substrate peptide. The increased proximity results in diffusion of the singlet oxygen to the acceptor beads and enhanced luminescence</td>
<td>Low enzyme consumption,</td>
<td>Sensitive to ambient light, assay data points can only be read once, sensitive to compound interference</td>
</tr>
<tr>
<td>Fluorescence polarization</td>
<td>Different rotational diffusion and rates of fluorescence depolarization of polarized light originating from fluorescein-labeled peptides,</td>
<td>Miniaturization potential, low enzyme consumption, bead-free</td>
<td>Low cost, antibody-free, antibody-dependent, sensitive to scattering, loss-of-signal format</td>
</tr>
<tr>
<td>ATP depletion</td>
<td>ATP consumption in the kinase assay is detected using luciferase activity as a reporter of remaining enzyme</td>
<td>Non-radioactive, antibody-free</td>
<td>Non-radioactive luminescence, antibody-dependent, loss-of-signal format</td>
</tr>
<tr>
<td>Fluorescence quenching</td>
<td>Binding of phosphorylated dye-labeled peptide to phosphate-binding Ca(^{2+})-modified nanoparticles quenches fluorescence</td>
<td>Antibody-free</td>
<td>Loss-of-signal format</td>
</tr>
<tr>
<td>Scintillation proximity assay</td>
<td>Capture of (^{33})P-labeled biotin-phosphopeptide by streptavidin-coated scintillant beads</td>
<td>Low cost, scalability in substrate, homogeneous reaction</td>
<td>Safety, limited ATP concentration range, poor signal:background</td>
</tr>
<tr>
<td>Time-resolved luminescence</td>
<td>Capture of biotinylated phosphopeptide on streptavidin-coated well plates followed by binding of lanthana-mediated antibody and luminescence detection</td>
<td>Low background</td>
<td>Antibody-dependent, non-homogeneous</td>
</tr>
</tbody>
</table>

of FRET, fluorescently labeled peptides can be employed in which the intrinsic fluorescence intensity is modified upon phosphorylation. For example, the fluorescent dye rhodamine 110 has suppressed fluorescence when two primary amino groups in its structure are covalently linked, for instance to two peptide sequences, to form a bisamide. If these peptides are phosphorylated, they are protected against proteolytic degradation but can be cleaved from the dye in their non-phosphorylated state by a suitable aminopeptidase leading to substantial dequenching of the dye [112]. Other sophisticated designs have employed a synthetic substrate peptide containing an artificial amino acid which exhibits Mg\(^{2+}\)-dependent chelation-enhanced fluorescence upon phosphorylation [113].

FRET-based intracellular reporters of real-time kinase activity have been developed which have the notable advantage of being able to conduct assays in a highly physiologically relevant environment [114]. These modular protein reporters consist of two distinct variants of green fluorescent protein (GFP) separated by an intervening kinase substrate domain and a phosphorylation recognition (SH2) domain. Provided that the GFP variants, specifically CFP and YFP, are within a sufficiently close distance, they can participate in energy transfer. Phosphorylation of the tyrosine within the substrate domain results in binding to the SH2 domain, bringing the GFP variants into close proximity for increased FRET.
1.4.4 Nanoparticle-enabled approaches to detect protein kinase activity

Whilst AuNPs have been tailored to detect kinase activity through enzyme-dependent agglutination/dispersion of the nanoparticles, several other nanoscale-specific phenomena have been applied to kinase detection. For example, two populations of magnetic nanoparticles, coated with a kinase substrate and complementary phosphopeptide-binding SH2 domains, can selectively coalesce in the presence of kinase activity and disperse in the presence phosphatase activity, as indicated by changes in NMR T$_2$ relaxation rates [115]. This non-optical approach is especially suited to strongly coloured or turbid samples such as cell lysates, but is somewhat limited the need for complex instrumentation and sophisticated engineering of heterogeneous populations of nanoparticles. Phosphopeptide detection has also been demonstrated using surface enhanced Raman spectroscopy (SERS) using the electric field-enhancing properties of gold and silver nanoparticles [116], providing a means for label-free detection. Whilst quantum dot photoluminescence has not been applied to detect kinase activity per se, a FRET-based competitive binding assay to identify molecules which bind to the conserved kinase ATP-binding site has been developed. Here a recombinantly expressed kinase construct bearing a QD-affinity peptide motif was self-assembled on the surface of the nanocrystal. A boron-dipyrromethene (BODIPY)-ATP analogue was designed to bind to the kinase-QD assembly through the enzyme’s ATP binding site, resulting in FRET from the QD to the BODIPY-ATP energy acceptor. Introduction of ATP-binding site competitors was envisioned to displace the BODIPY-ATP molecule from the QD assembly and result in diminished FRET. Control experiments, however, revealed that the system was susceptible to direct binding of the BODIPY-ATP molecule to the QD surface, thus significantly limiting the utility of the assay [117].

Distinct from their optical properties, the inherent curvature of nanoparticle materials can be utilised to create enhanced kinase assays to overcome limitations of other solid-phase enzyme assays. For example, kinase assays can be conducted using peptide substrates immobilised on a planar surfaces in chip format to allow large-scale parallel evaluation of different peptide sequences for biological activity [118] [119]. Some reports have suggested that a monolayer of gold nanoparticles coated with kinase substrates can serve as an efficacious alternative to substrates on flat surfaces, arguing that the curved nature of the particle allows a high peptide
loading density and affords enhanced steric accessibility for the enzyme.

1.4.5 Summary

The ability to quantitatively measure the activity of enzymes which mediate post translational modifications is of fundamental importance to drug discovery and to aid in our understanding of basic enzyme function. A host of fluorescence-based approaches have been developed to measure enzyme activity based on the use of organic dyes and fluorescent proteins. It is now becoming increasingly apparent that biofunctional nanomaterials can serve as powerful reagents for colourimetric- and fluorescence-based biological detection due to their size-tuneable and robust optical properties. Whilst they provide a persuasive route to avoid the use of radiolabeled tracers and photobleaching-prone fluorescent molecules, nanomaterial-based methods are not without limitations and face several challenges which currently limits their pervasive application in biosensing. For instance, reproducible methods of preparing large-scale quantities of purified nanoparticle-biomolecule conjugates of well-defined structure and stoichiometry are still in their infancy relative to other small-molecule probes for biological labeling. In addition, there is some ambiguity concerning the potential toxicity of nanomaterials which may arise due to their inherent physical dimensions or propensity to leach toxic constituent ions (e.g. Cd$^{2+}$) [120].

1.5 Overview

The goal of this thesis is to develop novel enzyme assays based on the unique optical and photoluminescent properties of semiconductor quantum dots and gold nanoparticles.

Chapter 2 seeks to develop an alternative method of protein kinase assay based on the aggregation-dependent optical absorption properties of colloidal gold nanoparticles. Some previously reported gold-nanoparticle based kinase assays have been limited by the use of weakly passivated nanoparticles prone to premature non-specific flocculation and the need for multi-step procedures or non-physiological surface-bound peptide substrates. In consideration of these caveats, an alternative method is proposed which employs protein stabilised AuNPs in a homogeneous assay whereby a synthetic peptide substrate analogue in solution can elicit nanoparticle aggregation in an enzyme-activity dependent manner.
Whilst quantum dots have been applied in a diverse range of energy transfer (FRET)-based biosensing systems, Chapters 3 and 4 seek to extend their use to the detection of protein kinases - a family of enzymes hitherto unexplored using such an approach. Here, quantum dots are rendered water soluble by means of ligand exchange with mercapto alkyl carboxylic acids and further derivitised with peptide substrates for two model protein kinases. Kinase catalysed phosphorylation of these QD-peptide conjugates is subsequently detected by means of an acceptor dye-labelled phospho-specific antibody. The resultant close proximity of the QD and acceptor dyes results in QD-dye energy transfer as revealed by steady-state and time-resolved photoluminescence measurements. This simple and homogeneous assay can be applied to measure inhibitor potency, suggesting that it may have utility in high-throughput drug-discovery applications. Chapter 5 builds upon this work to demonstrate that this overall sensing strategy has wider applications and can be readily generalised to detect functionally unrelated enzymes distinct from protein kinases. This is illustrated by the demonstration that histone acetyl transferases - a critical class of enzyme which regulates fundamental aspects of gene expression - can be detected using an analogous approach using QD-based FRET. This suggests that the enzyme assays developed here may have broad applicability and provides a generic platform for the detection of arbitrary enzyme-catalysed post-translational modifications.
Chapter 2

Kinase actuated immuno-aggregation of gold nanoparticles by bivalent peptide substrates

2.1 Introduction

The ability to impart biological functionality to inorganic nanostructures has permitted the development of a wide range of novel biological imaging and sensing probes. There are numerous examples where aggregation-dependent interparticle plasmon coupling exhibited by colloidal gold nanoparticles has been utilised to create convenient sensing platforms for biological analytes such as nucleic acids, proteins and other small molecules. These sensing systems typically employ gold nanoparticles which have been derivitised with specific recognition moieties such as DNA, nucleic aptamers, avidin/biotin and antibodies. The presence of the analyte results in either aggregation or dispersion of the nanoparticles and an accompanying change in the plasmon absorption spectra. This principle has also been extended to the detection of the activity of several enzymes such as proteases, protein kinases and other hydrolytic enzymes, owing to
the numerous advantages associated with nanoparticle-based sensing, relative to traditionally used isotopic and fluorometric approaches. Many of these nanoparticle-based approaches employ nanoparticle conjugates which serve as surrogate enzyme substrates and enzyme activity eliciting a change in the nanoparticle dispersion. Whilst the robust photostability and high extinction coefficients of gold nanoparticles provide numerous advantages to these systems compared to other techniques, this general approach has several important limitations. For instance, the preparation of gold nanoparticle-substrate conjugates can be technically challenging, requiring the use of cross-linking agents and additional purification steps. Although self-assembly and the strong gold-thiol interaction can be used to functionalise nanoparticles with substrate molecules and peptides the nanoparticle surface energy will be invariably modified and possibly render them susceptible to premature or irreversible flocculation [80]. In addition, the precise surface structure of the nanoparticle, local ion concentration and substrate packing density will also serve as key determinants of enzyme accessibility and it is possible that steric constraints will prevent the conjugate from acting as an accurate substrate mimic [121].

In light of these limitations, it was sought to design a gold-nanoparticle based enzyme sensing system for protein kinase activity which was not dependent on the use of surface-immobilised substrates. The system is based on the use of gold nanoparticles, functionalised with anti-phospho amino acid antibodies, designed to coalesce by means of a bivalent peptide substrate consisting of two tandem substrate sequences for a protein kinase separated by an oligoglycine spacer (Figure 2.1). Enzymatic phosphorylation of the two covalently-linked substrate sequences enables the peptide to cross-link the nanoparticles through immunorecognition by means of complementary antibodies bound to the surface of the nanoparticles, resulting in a red-shift in the particle absorption spectra due to interparticle plasmon coupling. It was envisioned that this approach should provide several benefits as the kinase substrate is free to diffuse in solution and not conformationally restricted due to nanoparticle immobilisation. This method should provide a more physiological mimic of natural substrates whilst endowing the assay with the photophysical benefits of gold nanoparticles and avoiding the use of radiolabels or fluorogenic tracers.

The tyrosine kinase Src was chosen as a model enzyme to develop the AuNP-based system in conjunction with a synthetic peptide based on previously developed substrates, owing to
previous kinetic and structural characterisation and implication in disease states [122] [108]. This peptide consisted of two copies of the enzyme substrate sequence linked by three glycine residues (H2N-IYGEFKKK-GGG-IYGEFKKK-CONH2) to provide additional conformational flexibility and spacing in order to favour interparticle cross-linking.

Gold nanoparticles were derivitised with antiphosphotryosine antibodies by i) electrostatic self-assembly or ii) thiol-directed immobilisation on the Au surface using a chemoselective cross-linker.

### 2.2 Materials and Methods

#### 2.2.1 Gold nanoparticles

40 nm citrate-stabilised gold nanoparticle colloids (9 x 10^{10} particles / mL) were purchased from British Biocell International (Cardiff, UK). Prior to further surface derivitization with antibodies, the particles were concentrated by ultrafiltration using a 10 KDa MWCO filtration device (4000 g) and resuspended in salt free 100 mM Na2PO4 (pH 7.5) to a final concentration of 1 nM.
2.2.2 Anti-phosphoamino acid antibody conjugation to gold nanoparticles

2.2.2.1 Electrostatic assembly

Monoclonal antiphosphotyrosine (PT-66) was conjugated to 40 nm gold nanoparticles using an electrostatic self-assembly procedure (Figure 2.2, i)) [123]. 200 μl of 40 nm AuNPs (1 nM) in borate buffer (pH 9.0) were incubated for 1 hr with different amounts of antibody to reach different final concentrations of antibody (1 to 24 μg/ml) in order to determine the minimum amount of antibody required to stabilise the sol against electrolyte-induced flocculation. Following incubation, NaCl was added to the AuNP/antibody solution to a final concentration of 100 mM and the UV/Vis absorption spectra recorded to detect the presence of particle aggregation, as indicated by a decrease in intensity at the sharp plasmon absorption band at ~520 nm and broadening in the spectra at wavelengths > 550 nm [96, 13].
2.2.2.2 Antibody oxidation and cross-linker conjugation

All reactions, unless otherwise stated, were conducted in 1.5 mL polypropylene centrifuge tubes (Eppendorf). Monoclonal anti-phosphoserine (clone PSR-45) and anti-phosphotyrosine (clone PT-66) were obtained from Abcam (Cambridge, UK) and Sigma respectively. In order to preferentially orientate antibodies on the colloids as to favour Fv region accessibility, a bifunctional dithiol-hydrazide cross-linker was employed (Figure 2.3, A) [124]. This scheme involves selectively modifying the antibody’s Fc region by through the selective reaction between oxidised carbohydrates and the hydrazide group of the cross-linker (Figure 2.3, B). Following which, the high-affinity multidentate gold-thiol interaction provides a means to preferentially orientate the antibodies on the nanoparticle surface. The antibodies were first buffer exchanged to 100 mM Na₂PO₄ (pH 7.5) to remove the sodium azide preservative from the storage medium owing to its propensity to react with aldehydes [125]. 10 µl of 100 mM sodium periodate (NaIO₄) dissolved in 100 mM Na₂PO₄ (pH 7.5) was then added to a 100 µl (1 mg/ml) antibody solution and allowed to react for 30 min at room temperature protected from light. The reaction was then quenched with the addition of 500 µl of PBS. A 20 µl aliquot was then tested for the presence of aldehydes by the addition of 60 µl 10 mg/ml Purpald® (4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole, Sigma) dissolved in 1 M aqueous sodium hydroxide. The presence of aldehydes was confirmed by the solution changing from colourless to deep purple in < 10 minutes. A 50 mM stock solution of cross-linker (dithiolaromatic-PEG₆-hydrazide, Sensopath Technologies, Bozeman, MT, USA) was prepared in ethanol (>99.5%, Sigma) and stored at -20°C prior to use. A 2 µl aliquot of the linker stock solution was then added to the oxidised antibody solution and allowed to react for 1 hr at room temperature. Following which, the antibody-linker conjugate was purified by four rounds of 10-fold concentration and dilution in 40 mM HEPES (pH 8.0) using a 10 KDa MWCO centrifugal filtration device (Millipore).

2.2.2.3 Antibody conjugation to gold nanoparticles

20 µl of the antibody-linker solution was added to 200 µl of the 1 nM 40 nm AuNPs in 100 mM Na₂PO₄ (pH 7.5), mixed gently by pipetting to ensure that the solution was homogeneous and allowed to stand for 30 mins at room temperature to allow linker immobilisation on the gold surface. Following which 100 µl of 10 µM PEG-thiol (CH₃O-PEG-SH) (5000 g/mol, Shearwater
Polymers) in 40 mM HEPES (pH 8.0) was added to the solution to passivate any exposed gold surfaces and allowed to stand for an additional 20 minutes. Alternatively, the PEG-thiol addition was substituted for 20 µl of 1% (w/v) BSA in 40 mM HEPES (pH 8.0). The resultant conjugates were purified by centrifugation (2000g, 10 minutes) and resuspension of the particle slurry in 0.1% (w/v) BSA in PBS three times and stored at 4°C before use.

2.2.3 Nitrocellulose blotting and silver enhancement assay

Nitrocellulose membranes (0.2 µm pore size, Sigma) were pre-rinsed in methanol and deionized water before use. 0.5 µl sample spots of were applied to the membrane and allowed to dry. The membrane was then blocked in 4% (w/v) BSA in PBS for 15 minutes, washed in PBS and incubated with a 1/25 dilution of antibody-conjugated gold nanoparticles in 1% (w/v) BSA in PBS for 3 hrs at room temperature with gentle agitation provided by an orbital shaker. The membranes were then rinsed with copious PBS and deionized water to remove unbound nanoparticles and subjected to silver enhancement. This involves deposition of metallic silver upon gold nanoparticles by the gold-catalysed reduction of Ag (I) ions by hydroquinone[126]. The washed membranes were incubated in a silver enhancement solution (British Biocell International) and allowed to incubate at room temperature for 15-20 minutes.
2.2.4 Peptide synthesis

Peptides can be readily synthesised using well-established solid phase methods developed by Merrifield [127]. In this approach, amino-acid monomers are sequentially incorporated into a growing peptide chain, chemically tethered to a polymeric solid-phase support resin, by formation of an amide bond between amine and carboxylic acid groups. The free N-terminal amino group of the resin-immobilised peptide reacts with excess activated ester derivative of the next amino-acid to be incorporated into the growing peptide chain. Potential self-polymerisation of the incoming amino-acid is prevented by temporarily blocking its N-terminus with a labile protecting group. Following amide formation, the N-terminus is subsequently deprotected, the resin washed with solvent to remove excess by-products and reagents, and the cycle repeated for the next amino-acid in the sequence. Other side-reactions during the synthesis are also prevented by blocking potentially reactive amino-acid side-chains with protecting groups that can be easily removed, under relatively mild conditions, at the end of the synthesis. This ensures that the amides of the peptide backbone are exclusively formed between the N- and C-termini of the amino-acids. Following completion of the synthesis, the peptide is released (cleaved) from the resin and the side-chain protecting groups simultaneously removed. Whilst this procedure can produce short peptides (<25 residues) of good purity, longer peptides or peptides of complicated sequence can pose more synthetic problems and recombinant bacterial expression systems are typically are a more suitable method of production.

C-terminal amide substrates and bis-substrate derivatives for Src (IYGEFKKGGGIYGEFKKK, IYGEFKKK) were synthesised by automated solid-phase peptide synthesis on an Applied Biosystems ABI 431A synthesiser. MBHA Rink Amide resin (0.63mmol/g loading) and Fmoc-protected amino acids were obtained from Novabiochem. All other solvents were obtained from AGTC Bioproducts (Hessle, UK). All peptides were synthesised on a 0.1 mmol scale with 10-fold molar excess of amino-acid monomers using HOBt/HBTU/DIEA activation in NMP and 1 h coupling times. Fmoc groups were deprotected using 20% piperidine in NMP. Following synthesis, the resin was washed with copious DMF and DCM in a fritted separating funnel and treated with a standard peptide cleavage/deprotection cocktail (5 mL trifluoroacetic acid (TFA) :triisopropylsilane (TIS) :water (95:2.5:2.5)) for 3 h. The volume of TFA was then reduced under a stream of N₂, the crude peptide precipitated and washed with ice-cold diethylether.
The peptides were purified by C-18 reverse phase HPLC using linear elution gradients of water-acetonitrile containing 0.1% TFA.

2.2.5 Enzyme assays

Enzyme reactions consisted of 10 units of Src, 10µM peptide substrate and 200 µM ATP in a total volume of 10 µl. The reaction mixture was incubated for 1.5 h at 30 °C prior to transferring an aliquot to a solution of antibody-coated gold nanoparticles (1 nM) to reach peptide concentrations from 4 pM - 500 nM.

2.2.6 Dynamic light scattering

Dynamic light scattering (also known as photon correlation spectroscopy) measures fluctuations in scattering intensity over time for particles in solution undergoing Brownian motion. Applying a correlation function in conjunction with the Stokes-Einstein equation:

\[ R_H = \frac{kT}{6\pi\eta D} \]  

where \( R_H \) is the hydrodynamic radius, \( T \) is temperature, \( D \) the diffusion coefficient, and \( k \) the Boltzmann constant, relates these fluctuations to the hydrodynamic radius of the particles, providing information on the size and size distribution of the particle dispersion. The hydrodynamic radius of the particles were measured on a Malvern Zetasizer Nano-ZS with a 633 nm laser source. All solutions were filtered using a 0.22 µm Millex® GS MF-Millipore MCE Membrane filter (Millipore, UK) prior to analysis in 1 cm pathlength disposable polystyrene cuvettes at room temperature. Measurements were collected with a minimum of 12 runs and analysed using the manufacturer’s software. Nanoparticle concentrations were on the order of 10 pM, diluted in PBS.

2.2.7 UV-visible absorption spectroscopy

UV-visible absorption spectroscopy measures the attenuation in intensity of incident light upon passing through a sample analyte relative to a standard. This attenuation is due the absorption of light by the sample arising from electronic transitions to an excited state and/or scattering
phenomena. The concentration of the analyte can be related to its absorbance, $A$, at wavelength $\lambda$ using the Beer-Lambert law, $A_\lambda = \log(I_0/I) = \varepsilon_{\lambda} c d$, where $I_0$ is the intensity of light transmitted through a reference, $I$ is the intensity of light which has passed through the sample, $\varepsilon_{\lambda}$ is the molar extinction coefficient ($M^{-1}cm^{-1}$), $d$ the absorption path-length and $c$ is the molar concentration of the absorbing species. UV-visible absorption spectra were carried out at room temperature on a Perkin Elmer LAMBDA 25 spectrophotometer (Waltham, MA) using 1 cm path-length quartz cuvettes.

2.3 Results and Discussion

2.3.1 Peptide synthesis

HPLC of the bivalent and monovalent control peptides revealed the presence of a single dominant elution peak suggesting the presence of a homogeneous synthesis product (Figure 2.4). The purified peptides were further analysed by MALDI mass spectrometry, yielding dominant m/z peaks at 1012 and 2177 for the control and bivalent peptides respectively corresponding to the predicted [M+H$^+$] masses (Figure 2.5).
Figure 2.4: Reverse phase HPLC traces of bivalent (A) and monovalent control (B) peptides. The single dominant peaks reflect the high purity of the peptide products ( > 98 %)
2.3.2 Antibody conjugation to gold nanoparticles

2.3.2.1 Electrostatic assembly

Preliminary studies were carried out to investigate the feasibility of producing AuNP-antibody conjugates based on direct electrostatic assembly through interaction with positively charged amino acid side chains on the antibody and the NP citrate stabilising layer [128]. This was achieved by titrating the antiphosphotyrosine antibody against 40 nm AuNPs (1 nM) at the IgG isoelectric point to determine the minimum amount of antibody required to stabilise the sol against electrolyte-induced aggregation initiated by the addition of NaCl to a final concentration of 100 mM. Whilst this is an appealing method owing to its relative simplicity it is limited by i) the inability to control the antibody orientation on the surface; ii) large quantities of protein are required to passivate the AuNP surface which may limit cost-effectiveness and iii) the non-covalent nature of the assembly may render the conjugate susceptible to ligand exchange and dissociation [101][124]. Nonetheless, initial efforts were focussed on preparing antibody-AuNP conjugates by electrostatic assembly. Antibody concentrations ≤8 μg/ml failed to provide any significant stabilisation, as indicated by a complete flocculation of the nanoparticles and loss of colourimetric signal (Figure 2.6). Even relatively high concentrations of antibody (24 μg/ml) failed to provide complete stabilisation, as supported in the UV-Vis absorbance spectra (Figure 2.7, A), suggesting that the nanoparticles may only be partially surface passivated at this
Figure 2.6: Salt-induced aggregation of 40 nm gold nanoparticles to determine minimum amount of antibody required to stabilise the colloid. The loss of colouration at lower antibody concentrations reflects complete particle flocculation in the presence of sodium chloride. Higher concentrations of antibody afford slightly greater resilience against particle flocculation, but the colour change from red to blue, relative to the control, suggests insufficient particle stabilisation.

In consideration of the poor stabilisation afforded by this method of bioconjugate preparation, the ambiguous surface orientation of the antibody and the large amounts of antibody required for nanoparticle stabilisation (i.e. poor cost-effectiveness) an alternative approach was investigated.

2.3.2.2 Dithiol-mediated assembly

In order to afford better control of antibody-nanoparticle orientation and to provide a more reproducible conjugation protocol, an alternative immobilisation procedure was utilised. This involved site-specific conjugation of dithiol appended cross-linker to the carbohydrate moieties of the antibody Fc region via hydrazide-aldehyde coupling and directed orientation of the antibody on the nanoparticle surface afforded by the bidentate thiol-gold interaction [124]. Assembly of the thiol-appended antibodies on the gold nanoparticle surface was confirmed from UV-vis spectra which demonstrated a small red-shift in the plasmon absorption peak from 525 nm to 529 nm (Figure 2.7, B), presumably due to a change in the nanoparticle local dielectric constant upon antibody binding [129]. In addition, no appreciable broadening in the absorption spectra at wavelengths > 600 nm was observed, suggesting that the addition of antibody did not induce substantial nanoparticle aggregation. Dithiol-mediated antibody assembly was also verified by
dynamic light scattering, revealing an increase in hydrodynamic radius from 45 nm to 75 nm relative to the uncoated citrate-stabilised AuNPs, consistent with the formation of an antibody layer on the nanoparticle surface. The bioconjugated gold nanoparticles did not show any visible sign of macroscopic aggregation for at least 1 month under storage at 4 °C.

Figure 2.7: UV-vis absorption spectra of antibody AuNP conjugates prepared by electrostatic absorption (A) and dithiol-mediated assembly (B) (normalised to plasmon absorption peak)

Figure 2.8: Dynamic light scattering analysis of citrate- and antibody-coated 40 nm AuNPs
Figure 2.9: UV-vis absorbance spectra of antibody-modified AuNPs in the presence of different concentrations of pY-BSA (normalised to plasmon absorption peak)

2.3.3 Bioconjugate binding to phosphorylated model proteins

In order to ensure that the antibody-AuNP conjugates retained functional antigen binding properties, control experiments were conducted using phosphotyrosine-conjugated BSA (pY-BSA) as a model phosphoprotein. It was anticipated that the protein should be able to act as an efficacious cross-linking molecule and cause aggregation of the anti-phosphotyrosine conjugated AuNPs, resulting in an observable change in the UV-vis absorption spectra due to interparticle plasmon coupling. To test this hypothesis, the antibody-AuNP conjugate was incubated with pY-BSA at different concentrations spanning three orders of magnitude. Under these conditions, no extensive red-shift, peak broadening or reduction in signal intensity was observed in the UV-vis absorption spectra, inconsistent with the model of pY-BSA-induced particle aggregation (Figure 2.9).

This behaviour can be explained by several potential mechanisms. For instance, the inherent pY-BSA structure and distribution of antibody-accessible phosphotyrosine residues on
the protein may render it unsuitable to effectively cross-link the antibody-AuNPs due to steric constraints. Unfortunately the manufacturer could not provide further information concerning the BSA-pY structure nor degree of phosphotyrosine loading on the carrier protein (personal communication). In addition, it may be possible that the particles do aggregate to some extent upon binding to pY-BSA, however the interparticle separation remains too great to affect a substantial colourimetric response [130]. Alternatively, the failure to observe a colourimetric signal may be due to a loss of antibody function upon immobilisation on the nanoparticle surface.

To investigate this final possibility, serial dilutions of pY-BSA or kinase reaction products were spotted on nitrocellulose membranes and incubated with the antibody-AuNP conjugate, followed by extensive washing and catalytic silver deposition on any surface-bound nanoparticles[131]. Successful binding was indicated by the development of grey spots on the membrane where the antigen was deposited. The protocol enabled rapid and convenient visual assessment of antibody binding and could detect down to 320 ng/ml (5 nM) concentrations of pY-BSA antigen, corresponding to 2.5 pg of total protein within 20 min. Furthermore, the sharp localisation of the enhancement restricted to the area in which the antigen was spotted and antigen concentration dependent enhancement intensity is indicative of the specificity of the antibody binding. A more quantitative analysis of antibody-AuNP binding may be afforded through the use of CCD based detection in conjunction with a more reproducible method of delivering uniform sample spots to the nitrocellulose membrane.

In summary, these results suggest that antibody-AuNP conjugates are functional and can successfully bind to target phosphoproteins however, the phosphotyrosine-conjugated BSA molecule was unable to elicit any colourimetric response in the nanoparticle system or produce any obvious signs of macroscopic precipitation. Although the pY-BSA molecule was unable to initiate nanoparticle aggregation, its structure should differ substantially from the kinase substrate peptides, thus it was assumed that these negative observations should not necessarily provide unequivocal evidence that the kinase substrate peptides designed here would not be able to initiate interparticle cross-linking.
Figure 2.10: Nitrocellulose membrane based immuno detection of phosphorylated proteins using silver deposition enhancement. A) Duplicates of different concentrations of phosphotyrosine BSA were immobilised on nitrocellulose membranes prior to treatment with antiphosphotyrosine-conjugated AuNPs and silver enhancement. B) Alternatively, aliquots of kinase reaction mixtures, containing different concentrations of the bivalent substrate peptide, kinase and +/- ATP were transferred to the membrane, followed by antibody-AuNP binding and silver enhancement, suggesting successful phosphorylation of the peptide and antibody binding.
2.3.4 Protein kinase activity assays

The bivalent peptide substrate for Src was incubated with the kinase followed by addition of aliquots of the enzyme reaction to the antibody-conjugated gold nanoparticles to reach different peptide concentrations. The reaction mixtures were then analysed by UV-vis absorbance and DLS. Should the peptides elicit particle aggregation in the presence of kinase activity, it was predicted that this could be readily detected by UV-vis absorbance and an increase in particle hydrodynamic radius or particle sedimentation. The normalised absorption spectra did not reveal any changes in the plasmon absorption peak position or peak broadening as a function of peptide concentration in the case of both the bivalent and monovalent control peptide, suggesting that no significant particle aggregation had taken place. This was also reflected by DLS experiments which did not provide any evidence of increase in particle hydrodynamic radius or aggregation. It was observed however, that higher concentrations of peptide could induce a subtle red-shift in the nanoparticle absorption spectra after ∼3 hr, with the plasmon absorbance peak shifting from 532 nm to 534 nm (Figure 2.11). This occurred in both the case of the bivalent and control peptide substrates or control experiments in the absence of ATP, suggesting that this was likely due to non-specific effects. For instance, the peptides or enzyme may displace the nanoparticle-bound antibodies or surface-adsorbed BSA molecules resulting in a decrease in colloidal stability over time. This may be due to some of the antibodies being bound to the nanoparticle surface by more labile electrostatic absorption mechanisms as opposed to the higher-affinity thiol-directed immobilisation. In addition, the presence of a cluster of thiol-bearing cysteinyl residues within the Src kinase domain, as indicated by the sequence of the protein and high resolution crystal structure [132], may contribute to this inherent instability by providing a high affinity binding motif to compete for the AuNP surface.
2.4 Conclusions and Future Work

This chapter has described the attempt to develop novel peptide substrates which can selectively induce the aggregation of antibody-modified gold nanoparticles in response to protein kinase activity to provide a convenient colourimetric signal. Whilst it was not possible to demonstrate successful detection of kinase activity in this case, several important lessons were learned here which helped guide the development of additional nanoparticle-based enzyme detection strategies, as detailed in the remainder of this thesis.

As part of the goal of realising rapid, simple and convenient nanoparticle-based enzyme detection it was concluded that the approach explored here would not provide an appropriate solution for kinase screening for several reasons. For instance, whilst the original intention of this study was to introduce peptides which could circumvent the use of immobilised peptides to
afford more physiological substrate analogues and simplicity in assay design, this approach necessitated reasonably sophisticated antibody modification procedures, thus the predicted benefits of the system were counteracted by the complexity and cost introduced by additional bioconjugation steps. In addition the assay is reliant on a complex interplay of numerous biomolecule interactions with varying affinities and rates of association/dissociation. Some of the potential interactions may indeed be antagonistic to the operation of the assay - for example, the bisphospho peptide may be unable to mediate interparticle immuno-aggregation due to its local concentration favouring an intraparticle interaction (i.e. binding to two antibodies on the same nanoparticle) or steric repulsion providing too great a kinetic barrier and preclude interparticle collisions required to initiate particle aggregation [88]. Furthermore, the number of potential interactions at play presents a broad and complicated parameter space to optimise and may result in narrow windows of operation in terms of reagent concentrations. For example, a previously reported AuNP-based lactamase sensor, using a similar cross-linking principle, exhibited a profound dependence on nanoparticle concentration and only exhibited enzyme-responsive aggregation behaviour within a AuNP concentration range spanning from 1.3-3 nM [133]. Other designs could be investigated to increase the probability of peptide phosphorylation driving particle aggregation by increasing the effective valency of the peptide. For example, as opposed to the use of a bifunctional peptide, capable of cross-linking a maximum of two individual nanoparticles, a dendrimeric peptide structure containing multiple potential binding sites could provide a greater propensity to initiate large-scale particle aggregation and could be realised by conjugating an appropriate peptide substrate to a branched dendrimer core [134].

The observed particle red-shifts at longer time scales were likely due to non-specific interactions and/or displacement of the nanoparticle passivating protein layer. This could be limited using more robust ligand coating shells less susceptible to ligand exchange, such as UV cross-linked diacetylene motifs, which could be subsequently conjugated to the antibody molecules [100]. In order to minimise the possibility of some of the antibodies becoming bound to the nanoparticle surface via labile electrostatic absorption as opposed to thiol-mediated binding, it may also be possible to develop alternative cross-linking agents and chemoselective antibody coupling strategies. For example, the nanoparticles could initially be derivitised with a homobifunctional hydrazide followed by selective coupling to the oxidised antibody [65].
It could, however, be possible to greatly simplify the system, remove the need for sophisticated bioconjugation and abrogate the need for phospho-specific antibodies. For example, the peptides designed here could be used in conjunction with streptavidin-coated AuNPs and a $\gamma$–biotin derivative of ATP [13], with kinase activity resulting in bis-biotinylation of the peptide and subsequent streptavidin-AuNPs cross-linking. This could provide a convenient homogeneous assay without need for additional nanoparticle separatory or purification steps, assuming that the enzyme can utilise the biotinyl-ATP analogue and that the size of the nanoparticle aggregates are commensurate with an appreciable colourimetric response.
Chapter 3

Preparation of Peptide-Coated Semiconductor Nanocrystals For Kinase Sensing and Cellular Imaging

3.1 Introduction

Chapters 3 and 4 detail the development of an alternative approach to protein kinase biosensing based on the use of photoluminescent semiconductor nanocrystals as FRET donors, with Chapter 3 describing the development of new QD-peptide conjugates and Chapter 4 demonstrating their utility in the detection of two prototypal tyrosine kinases, Src and Abl [114, 108].

Fluorescence-based detection offers several advantages compared to absorbance measurements due to increased sensitivity and the fact that FRET sensing can afford a dual parameter response (i.e. changes in both donor and acceptor fluorescence intensity at two distinct wavelengths). This provides a robust ratiometric signal which assists in accounting for inter-experiment variability, to which single-wavelength absorption or fluorescence measurements are particularly prone.
3.1.1 Overview of experimental design

Numerous QD assays for enzymatic activity are based on energy transfer owing to the numerous benefits associated with QDs as energy transfer donors. These assays, however, have been largely restricted to hydrolytic enzymes, such as proteases and bacterial β-lactamases [4, 135, 65, 136]. Here, enzymatic activity drives the dissociation of an energy transfer acceptor, such as an organic dye or gold nanoparticle quencher, from the immediate vicinity of the QD resulting in changes in non-radiative energy transfer from the QD to acceptor. Nonetheless, hydrolytic enzymes are but a small sub-class of the diverse families of enzymes which mediate protein post-translational modifications. For example, there are a wide range of enzyme-mediated modifications mediated by enzymes which operate with fundamentally different mechanistic details to hydrolytic enzymes. Such enzymes catalyze the addition of modifying groups to various amino acid side chains to impart new functionality upon the target protein. It is thus difficult to readily reconfigure QD assays for hydrolytic enzymes to examine other important enzyme-mediated modifications without fundamental redesign of the system. Therefore, it was sought to develop a new class of assay which could be applied to study enzyme reactions which result in the formation of covalent substrate adducts, as opposed to hydrolytic cleavage events.

As a model system and owing to their central role in disease, it was chosen to develop an assay towards measuring the activity of protein tyrosine kinases which employed the photophysical properties of QDs.

The QD is conjugated to a protein kinase substrate peptide by means of a hexahistidine (His\textsubscript{6}) motif which self-assembles on the ZnS surface by virtue of a multivalent interaction afforded by consecutive imidazole side-chains [137]. This QD-peptide conjugate is acted upon by a corresponding protein kinase in the presence of ATP, resulting in phosphorylation of tyrosine residues. This is subsequently detected by means of immunorecognition-driven binding of a complementary anti-phosphotyrosine antibody conjugated to fluorescent dye molecules (Figure 3.1). Provided that the QD core is in sufficiently close proximity to the dyes, energy transfer (FRET) can take place, as manifested by an increase in dye-specific emission and decrease in QD-specific emission. It was predicted that several features of this system should be conducive to efficient FRET. For instance, bioconjugate preparation based on self-assembly of the peptides directly on the surface of the nanocrystal ensures that the overall dimensions of the complex...
Figure 3.1: QD-based FRET immunoassay for tyrosine kinase activity (one peptide/QD shown for clarity). Dye-labeled antibody recognizes the kinase phosphorylated peptide, resulting in QD-dye energy transfer and is manifested as a simultaneous increase and decrease in dye-specific and QD-specific emission respectively.

are relatively compact (Figure 3.1, 1)), enabling the acceptor fluorophores to come within close proximity of the QD FRET donor to favour efficient FRET - in contrast to other means of QD-bioconjugate preparation which rely on bulky streptavidin or polymer coatings [61, 4]. In addition, each QD FRET donor can potentially interact with multiple FRET acceptors due to the multivalent nature of the QD-peptide assembly and the antibodies’ capacity to conjugate to several acceptor dyes, affording a high overall acceptor:donor ratio and enhanced energy transfer (Figure 3.1, 2)) [3].

3.1.2 Peptide conjugated quantum Dots

Since the first demonstrations of antibody labeling of semiconductor nanocrystals (quantum dots, QDs) for cellular imaging in 1998 [138], significant technical advancements have been made to enable the conjugation of a variety of other biological molecules. Improved synthetic procedures in the literature and the availability of commercial sources of QDs with a range of surface coatings has greatly facilitated the development of biological applications of QDs. As part of the eventual goal of utilising QD bioconjugates for FRET-based enzyme sensing, it was sought to investigate a range of approaches to prepare peptide-conjugated QDs. This chapter describes the solubilisation and bioconjugation of CdSe/ZnS core-shell QDs to a range of synthetic peptides corresponding to protein kinase substrate sequences.
Endowing QDs with the biological and chemical functionality of peptides results in the ability to use their unique photoluminescent properties to interrogate a wide range of cellular and biological processes. For example, NIR-emitting QDs modified with cell-targeting peptides, such as the cyclic-RGD integrin binding motif, can be used for non-invasive in vivo small animal imaging of tumor vasculature [139]. Oligoarginine-tagging results in cellular internalisation of QDs and coating with enzyme-hydrolyzable peptide sequences affords a range of proteolytic sensing systems [64, 3, 38, 140]. Several strategies to successfully interface QDs with peptides have been explored, each with associated advantages and limitations. We sought to develop a peptide:QD conjugation procedure which:

- could be applied to commercially available sources of QDs in order to circumvent complicated synthesis protocols requiring potentially hazardous precursors;
- did not require the use of cross-linking agents to simplify subsequent purification;
- did not require substantial expertise in chemical synthesis;
- afforded reasonable control of QD:peptide stoichiometry with predictable peptide orientation on the QD surface;
- produced compact conjugates of relatively small hydrodynamic radii, owing to final intended applications in for energy transfer (FRET) based sensing.

To this end, commercially available CdSe/ZnS QDs were rendered water soluble by base-assisted ligand exchange with a hydrophilic low molecular weight thiol and subsequently modified with peptides using a self-assembly strategy. Steady-state and time-resolved spectroscopy in addition to gel-electrophoresis were implemented to characterise the photoluminescence and structural details of the QD-peptide systems. In addition, as part of ongoing work and in order to validate the biological activity of these QD-peptide systems, some of these conjugates were applied to a simple immunoassay and cell-imaging.

Nanobioconjugates prepared by self-assembly procedures are highly advantageous compared to covalent coupling methods owing to associated speed and convenience. Quantum dots have been subject to modification with peptides using various self-assembly procedures. For instance, proteins appended with a sequence of contiguous positively charged amino acid residues
or cationic leucine zipper domains can self-assemble on anionic QDs by simple electrostatic association [141] [38], however the generality of this method may be limited, particularly in the case of inherently cationic peptides and proteins. In addition, dative bonding between thiol groups of cysteine residues and surface sulphur atoms has been used to conjugate protease substrates and cell-penetrating peptides on QD surfaces [142] [143]. This does not provide a high-degree of control over biomolecule:QD stoichiometry and requires exposing the peptides to high concentrations of organic solvent and would therefore be a poor conjugation strategy for some proteins with sensitive three-dimensional structure. It has also been demonstrated that oligohistidine-terminated peptides and recombinant proteins can self-assemble upon the surface of dihydrolipoic acid (DHLA) capped QDs due to a multivalent metal-affinity coordination of histidine imidazole groups to surface Zn atoms [144, 145, 117]. This is a persuasive option as the conjugation step is performed in aqueous solution and affords some degree of control over final QD:biomolecule ratio. A drawback of this method is the reliance on prior solubilisation of the QDs using DHLA - a reasonably complicated multistep procedure requiring high concentrations of QD and reduced DHLA [41]. Nonetheless, hexahistidine-appended proteins can also self-assemble on anionic phospholipid-coated nanocrystals, as suggested in FRET studies using recombinant fluorescent proteins [144]. It was unclear, however, whether the assembly process was due to a direct interaction between the protein and inorganic shell of the QD or through the hydrophilic surfactant coating. An alternative method of conjugation has been described based upon divalent cation-dependent assembly of hexahistidine-tagged proteins on QDs coated with a carboxylic acid functionalised stabilising polymer [146]. Similarly, QDs can be derivitised with a nickel-triacetic acid motif, to which hexahistidine-tagged biomolecules can bind through high-affinity chelation [147]. These are appealing methods owing to their simplicity, but are reliant upon bulky polymeric stabilisers, resulting in a large hydrodynamic radius which would preclude efficient FRET [46].

To develop new QD-peptide conjugates for enzyme sensing, and in consideration of the available options, a modified conjugation method was sought which could utilise simple-self assembly in conjunction with hydrophilic quantum dots prepared by straightforward chemical derivitisation to afford stable biomolecule-nanocrystal conjugates. Here, commercially available red-emitting (605 nm emission maximum) CdSe/ZnS core/shell QDs were rendered water soluble
by simple base-promoted ligand exchange with a hydrophilic low molecular weight thiol and further modified with hexahistidine-appended peptides (Figure 3.2). These bioconjugates can be applied to simple immunoassays and new methods to screen enzyme activity, as detailed in the remainder of this thesis.

Figure 3.2: Schematic representation of experimental strategy to prepare peptide-QD conjugates. Hexahistidine-mediated assembly of peptides on ZnS surface of mercaptopropionic acid (MPA) stabilised QDs. Hydrophobic stabilising surfactant is replaced with MPA by means of base-promoted ligand exchange. Multivalent imidazole-ZnS interactions provided by the consecutive histidine residues afford stability against peptide dissociation.

3.2 Materials and Methods

3.2.1 Quantum dots (QDs)

All solvents and reagents, unless stated were obtained from Sigma-Aldrich. Core/shell CdSe/ZnS quantum dots (605 nm emission maximum, 1.0 μM stock) in decane were obtained from Invitrogen. Prior to use, the QDs were transferred to a polypropylene centrifuge tube and flocculated with the addition of a 5-fold volume of 3:1 MeOH/iPrOH. The QDs were centrifuged at 6000 g for 2 min, the supernatant decanted and the QDs resuspended in CHCl₃ to a final concentra-
QDs were visualised during benchtop manipulations under 365 nm illumination provided by a hand-held UV lamp.

### 3.2.2 MPA exchange

10 ml of CHCl$_3$ and 500 $\mu$l of mercaptopropionic acid (MPA) were added to 1 g of tetramethyl ammonium hydroxide pentahydrate (TMAH) in a 50 mL polypropylene centrifuge tube, mixed vigorously by vortexing for 30 s and allowed to stand for 30 min at room temperature. The two phase mixture resulting from the TMAH water of crystallisation was centrifuged (4000 g, 5 min) and the lower organic phase (approx. 90 % of the total volume) decanted. 1 ml of 100 nM QDs in CHCl$_3$ was added to the organic phase and the mixture allowed to stand at room temperature protected from light for 40 hr. Initially, the solution exhibited homogeneous photoluminescence under UV illumination. Following incubation, however, the QDs formed distinct photoluminescent droplets floating on the surface of the CHCl$_3$ phase. The droplets were washed three times with 10 ml CHCl$_3$ and extracted with 10 ml of 10 mM borate buffer (pH 9.0) with the QDs seen to transfer completely into the aqueous phase. The aqueous phase was then subjected to three cycles of ten-fold concentration/dilution using 10 KDa MWCO ultrafiltration units (Millipore) at 4000 g for 10 min. The MPA-capped QDs were then stored in the dark at 4$^0$C. The concentration of the QDs was determined from the absorption intensity of the absorption onset peak in the UV-visible absorption spectra ($\epsilon_{596} = 650,000$ M$^{-1}$cm$^{-1}$, manufacturer’s information).

### 3.2.3 Synthesis of octylamine-modified polyacrylic acid (O-PAA)

An amphiphilic polyacrylic acid (PAA) derivative was prepared using a previously reported procedure [148]. 2.16 g of polyacrylic acid (Sigma, free acid form, 2000 g/mol, supplier’s information) and 2.85 g of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) was dissolved in 20 mL of DMF in a 100 mL round bottom flask equipped with a magnetic stir plate. 1.6 mL of octylamine was added dropwise over the course of 10 minutes and the clear solution was mixed overnight at room temperature under nitrogen. This molar ratio results in 40 % octylamine substitution of the total PAA carboxylic acid groups. The progress of the reaction was monitored by thin layer chromatography and ninhydrin staining. When the reaction
was complete, as indicated by a negative ninhydrin test, 75% of the DMF was removed using a centrifugal evaporation system (Genevac HT-4, Gardiner, NY). The residue was transferred to a polypropylene centrifuge tube and mixed with 10 mL of acetone and 25 mL water. The precipitated product was recovered by centrifugation (4000 g, 5 min) and washed with 25 mL water three times. The solid was dissolved in 40 mL ethyl acetate in a 100 mL round bottom flask with gentle heating at 40°C. 6.1 g TMAH in 25 mL H₂O was added and the mixture stirred for 15 min. The aqueous layer was collected using a separating funnel and acidified to pH 2.0 using 2N HCl. The cloudy precipitate was collected by centrifugation (3000 g, 5 min) and washed with 50 mL H₂O three times. The residue was redisolved in 50 mL ethanol and dried under vacuum at 60°C to afford a pale yellow sticky solid. 100 µl of a 100 nM QD solution in chloroform was added to 1ml of a 1 mg/ml solution of O-PAA in CHCl₃, the solvent evaporated and the QDs redispersed in 1 ml 50 mM borate buffer (pH 9.6). The solution was passed through a 0.45 µm syringe filter (Millipore) and purified by ultrafiltration through a 50 KDa cut-off centrifugal filtration device (Millipore).

### 3.2.4 DHLA synthesis

Dihydrolipoic acid was prepared by reduction of thiotic acid [41]. 3 g of thiotic acid (Sigma) was dissolved in 60 mL aqueous 0.25 M sodium bicarbonate in a 100 mL round bottom flask on ice. 600 mg of sodium borohydride (NaBH₄) was added in 10 mg aliquots over a 10 min period with continuous stirring of the solution. After one hour, the yellow solution had turned colourless. 50 mL of ethyl acetate was added and the mixture acidified to pH 1.0 using 2 N HCl. The organic phase was collected using a separatory funnel, dried with magnesium sulphate, filtered and the solvent removed under vacuum to afford a pale yellow oil which was stored at -20°C before use.

### 3.2.5 Peptide synthesis

All reagents were obtained from Novabiochem. Peptides based on known substrate sequences for Src (Ac-IYGEFKKKHHHHH-CONH₂) and Abl (H₂N-AEIYAAPFAEEHHHHH-CONH₂), and complementary tyrosine/phenylalanine substitution peptides (Ac-IFGEFKKHHHHH-CONH₂, H₂N-AEIIFAAPFAEHHHHHH-CONH₂) were prepared using a standard Fmoc coupling strategy on an Aapptec Apex 396 combinatorial peptide synthesiser. 1 mM stock solu-
tions of Fmoc side-chain protected amino acid monomers and equimolar quantities of HOBr were dissolved in DMF before use. 1 hr coupling reactions employed 4-fold excess of amino acid monomer using N, N'-diisopropylcarbodiimide as a condensing agent. Deprotection steps and cleavage reactions were conducted as previously described. Peptides were purified to > 98% by C-18 semi-preparative HPLC (Varian, Walnut Creek, CA) using linear elution gradients of H₂O/acetonitrile containing 0.1% TFA. The identity of the peptides was verified by liquid chromatography/mass spectrometry (LC/MS) (Agilent 1100, Santa Clara, CA).

<table>
<thead>
<tr>
<th>Peptide Sequence</th>
<th>Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Src) Ac-IYGEFKKKHHHHH-CONH₂</td>
<td>1876.1 gmol⁻¹</td>
</tr>
<tr>
<td>(SrcF) Ac-IFGEFKKKHHHHH-CONH₂</td>
<td>1860.0 gmol⁻¹</td>
</tr>
<tr>
<td>(Abl) H₂N-EAIYAAPFAEEHHHHH-CONH₂</td>
<td>2032.2 gmol⁻¹</td>
</tr>
<tr>
<td>(AblF) H₂N-EAIFPFEEHHHHH-CONH₂</td>
<td>2016.2 gmol⁻¹</td>
</tr>
</tbody>
</table>

Table 3.1: Predicted masses of peptides used throughout this study

3.2.6 Peptide conjugation to QDs by metal-affinity driven self-assembly

1 mM stock solutions of peptide were prepared in deionized water and stored at -20°C before use. Stock solutions were diluted in 10 mM sodium borate buffer (pH 9.6) to 1μM. 20 μl aliquots of 100 nM MPA-capped QDs were incubated with different volumes of peptide to achieve different molar ratios of peptide:QD, from 0-50:1 and the solutions mixed rapidly to ensure homogeneity.

3.2.7 Agarose gel electrophoresis

1% (w/v) agarose gels were prepared by dissolving routine use agarose powder (Sigma) in Tris-acetate-EDTA (TAE buffer, 40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, pH 7.8) with microwave-assisted heating. 20 μl aliquots of MPA-capped QDs (100 nM) in 10 mM sodium borate (pH 9.6) were incubated for 1 hr with different volumes of peptide solution (1 μM) to achieve peptide:QD ratios ranging from 0-50:1. Glycerol (100%) was then added to the solutions to reach a final concentration of 5% (v/v) prior to well loading. The peptide conjugates were then run on agarose gels in TAE buffer for 30 mins at 10 V/cm and imaged under 365 nm illumination.
3.2.8 UV-Vis absorbance spectroscopy

UV-visible absorption spectra were carried out at room temperature on a Perkin Elmer LAMBDa 25 spectrophotometer (Waltham, MA) using 1 cm path-length quartz cuvettes.

3.2.9 Steady-state photoluminescence

Steady-state photoluminescence emission spectra were recorded at room temperature on a Jobin Yvon FluoroMax-4 Flourimeter using 50 µl quartz three-window fluorescence cuvettes. 400 nm excitation and 5 nm excitation/emission. The acquired spectra were corrected for variations in lamp and detector efficiency with files from Jobin Yvon. Alternatively, fluorescence intensity measurements were carried out in black 96-well fluorescence microplates (Cornig, Rochester, NY) on a Gemini XS microplate reader (Molecular Devices, Sunnyvale, CA).

3.2.10 Measurement of quantum yield

Quantum yields ($QY$) of QDs were calculated relative to absorption-matched dilutions of sulphorhodamine 101 in ethanol as a reference ($QY = 0.90$) according to:

$$QY_{QD} = QY_R \times \frac{I_{QD}}{I_R} \times \frac{A_R}{A_{QD}} \times \frac{\eta^2_{QD}}{\eta^2_R}$$

where $I$ is the integrated luminescence intensity, $A$ is absorbance intensity at the excitation wavelength (400 nm) and $\eta$ is the refractive index of the QD and reference ($R$) solutions [149].

3.2.11 Time-resolved photoluminescence

Time-resolved single photon counting (TCSPC) provides information on the excited state lifetime of fluorescent molecules. In essence, an ensemble of fluorophores is excited with a pulsed excitation source and the arrival time of single photons emitted from the sample is recorded, with the process repeated many times to build a histogram of photon arrival times. In many scenarios the excitation pulse-width is on a comparable time scale as the sample lifetime and, as such, the observed decay is a convolution of the excitation pulse (instrument response function) and the sample impulse response. Deconvolution is achieved by considering the excitation pulse as a series of infinitely sharp $\delta$-functions of different amplitudes resulting in a measured signal which
can be expressed as sum of many individual decays with different amplitudes and start times. In practice, the instrument response function can be readily measured by recording the decay of a zero-lifetime sample, such as a scattering sample of dilute colloidal silica. TCSPC can provide insight into the photoluminescent lifetimes of QDs, which exhibit lifetimes relatively longer than those of standard organic fluorophores (10-100 ns) and size- and wavelength-dependent multi-exponential decay profiles, due to the inherent QD size distribution and dynamic non-radiative decay channels [150][151][152].

Time-resolved PL decays were collected on a Jobin Yvon Fluorolog TCSPC spectrophotometer using a 200-ps 440 nm LED modulated at 1 MHz as an excitation source on samples with an absorbance of < 0.1 at 440 nm. 10,000 count coaxial delay of 65 ns, time-to-amplitude converter (TAC) range of 50 ns was employed. The instrument response function decay curve was measured using a dilute aqueous solution of colloidal silica (Ludox). Average sample lifetimes were calculated by fitting the measured decays to a convolution of the instrument response function and a double-exponential decay. Average lifetimes ($\bar{\tau}$), were calculated according to [149]:

$$\bar{\tau} = \frac{\alpha_1 \tau_1^2 + \alpha_2 \tau_2^2}{\alpha_1 \tau_1 + \alpha_2 \tau_2}$$

3.2.12 QD conjugation to antibodies for phosphoprotein detection and cell labeling

The purified B1 immunoglobulin binding domain of Streptococcal protein G (GB1), expressed with an N-terminal hexahistidine tag was a gift from Ms Cheryl Goldbeck (Lawrence Berkeley National Laboratory) [153]. In a final volume of 100 µl, 50 nM QD-MPA in 50 mM borate buffer (pH 9.0) was incubated with a 30-fold molar excess of GB1 for 30 min at room temperature. Following which, excess GB1 was removed by 3 rounds of 10-fold concentration/dilution using a 50 kDa MWCO centrifugal ultrafiltration unit (Millipore, 4000 g, 8 min) and resuspended in 50 µl PBS containing 0.1 % (w/v) BSA. An aliquot of monoclonal antiphosphotyrosine (clone PT-66, Sigma Aldrich) or anti-actin (clone YOL1/34, Abcam) was added to the QD-GB1 conjugate (antibody:QD ratio 4:1) and incubated for 1 hr at room temperature and used without further purification.
3.2.13 Heterogeneous immunoassay and cell labeling

3.2.13.1 Immunoassay

Nitrocellulose membranes (0.2 µm pore size, Sigma) were pre-rinsed in methanol and deionized water before use. Serial dilutions of phosphotyrosine-modified BSA (Sigma) (0.5 µl) were spotted on the membrane and allowed to dry for 1 min before blocking with 4 % (w/v) BSA in PBS for 30 min. The membranes were then incubated with a 1 % (w/v) BSA-PBS solution of QD-GB1-antibody (10 nM QD) for 2 hr at room temperature followed by washing with copious PBS.

3.2.13.2 Cell labeling

Mouse embryonic NIH 3T3 fibroblasts (ATCC) were grown to 70% confluence on poly-D-lysine coated glass-bottom microscopy dishes (MatTek, Ashland, MA) in high-glucose DMEM containing 10 % fetal bovine serum. The cells were rinsed with copious PBS and fixed with 4% (w/v) paraformaldehyde in PBS for 10 min and permeabilised/blocked with 0.1% (v/v) triton-X100/4% BSA (w/v) in PBS for 30 min. The fixed cells were incubated with 10 nM QD-GB1-antitubulin for 3 hr at room temperature. Images were acquired on an Olympus IX-81 inverted microscope equipped with a 75W Xe lamp, a PLAPON 60x 1.45NA oil-immersion lens, and an Andor iXon+ EMCCD. Exposure times were 100 ms and all images were captured under identical camera conditions. Images were acquired through a Cy5 filter set using a Xe lamp and DAPI excitation filter (Semrock). Images were collected and analyzed using Slidebook 4.1 (Intelligent Imaging Innovations). All images were background corrected using a cell-free area from the first image and set to identical dynamic intensity ranges with a linear contrast adjustment.

3.3 Results and Discussion

3.3.1 Peptide synthesis

The HPLC-purified and lyophilised peptides were subjected to LC-MS, yielding molecular ion peaks within 1 mass unit of their predicted molecular weights thus verifying their identity (Figure 3.3).
3.3.2 Phase transfer of QDs

It is possible to perform biomolecule conjugations on commercially available water-soluble QDs using various covalent cross-linkers or streptavidin/biotin interactions, however these approaches have certain limitations with respect to energy transfer-based sensing. Covalent cross-linking to polymer-coated QDs often involves extensive processing and purification procedures, increasing the likelihood of loss of material. Although streptavidin-biotin mediated self-assembly of nanoparticle conjugates using streptavidin-coated QDs is reasonably straightforward, the large dimensions of streptavidin contributes to a considerable distance between the QD core and acceptor dye and may preclude high-efficiency FRET. Thus, it was sought to use an approach based on peptide self-assembly directly to the ZnS surface of the QD in order to abrogate the use of cross-linkers, facilitate purification and minimise the final hydrodynamic radius of the conjugate. To this end, we aimed to transfer commercially available QDs, soluble only in non-polar organic solvents, into aqueous solution. This was achieved by base-assisted ligand exchange of the native QD surfactant coating with MPA (Figure 3.4). This procedure employs TMAH to...
deprotonate the MPA thiol which in turn enhances its reactivity towards the ZnS surface [37]. Quantum yield and colloidal stability are often substantially diminished when thiols are used to solubilise QDs [154].

Dithiols can provide improved colloidal stability, however high concentrations of QD and dithiol are required and such procedures are not commensurate with straightforward bench-top manipulations [41]. The approach used here, however, could be conducted under ambient conditions, and yielded stable QD dispersions which did not show any signs of macroscopic aggregation when stored at room temperature or at 4°C over a period of several months and high-speed centrifugation at 30,000 g for 10 min did not result in any visible pelleting, verifying that the QDs were well dispersed. Furthermore, whilst photooxidation may lead to instability in some mercaptocarboxylic acid coated QDs [155], the MPA-capped QDs did not show any noticeable propensity to aggregate under ambient lighting conditions over time. The efficiency of the phase-transfer was typically c.a. 85% as determined UV-vis absorbance.

\[
\begin{align*}
\text{HS-} & \quad \text{OH} \\
\text{MPA} & \quad \text{TMAOH} & \quad \text{CHCl}_3 & \quad \text{Me}_4\text{N}^+ \\
\text{O} & \quad \text{Me}_4\text{N}^+ \\
\text{+ lipophilic QDots} & \quad \text{CHCl}_3 & \quad \text{Water-soluble MPA-capped QDots}
\end{align*}
\]

Figure 3.4: Base-assisted phase transfer of quantum dots to aqueous solution
3.3.3 Optical properties of MPA-capped QDs

UV-vis spectroscopy indicated that the position of the absorption onset 596 nm did not change in position between the MPA-capped QDs or the as-received hydrophobic surfactant-coated QDs. In light of this observation, it was assumed that the molar extinction coefficients of the MPA and TOPO capped quantum dots at the first exciton were equivalent and was used for all subsequent concentration calculations. As a comparison, an aliquot of QDs was rendered water soluble by encapsulation within an interdigitating amphiphilic polymer, octylamine-modified polyacrylic acid (O-PAA) [148] and exhibited a subtle red-shift in the absorption onset to 601 nm, possibly reflecting partial leakage and delocalisation of the exciton wave function into the polymer shell (Figure 3.6) [29]. There was a slight decrease in quantum yield in the case of the MPA-capped QDs from 0.55 to 0.44 and a slight decrease in absorption intensity at shorter wavelengths < 400 nm, relative to surfactant-coated hydrophobic QDs, consistent with previous reports (Figure 1.6) [37]. This may reflect specific electronic effects upon thiol absorption to the QD surface the loss of some population of QDs during the phase transfer process [156].
Figure 3.6: UV-Vis absorbance of surfactant coated QDs in CHCl$_3$, MPA-capped QDs in aqueous buffer (10 mM borate, pH 9.6) and amphiphile-coated QDs (40% octylamine modified polyacrylic acid, O-PAA) in buffer (normalised to first exciton absorption peak)

Steady-state photoluminescence suggested that the emission maximum peaks, centered $\sim$605 nm, and emission peak widths of the QDs with different coatings to provide solubility in aqueous solvent remained essentially unchanged from the hydrophobic QDs (TOPO coated) in chloroform (Figure 3.7). Again, some red-shift in emission maximum was observed in the case of the polymer-stabilised nanocrystals, in line with other studies which have used amphiphile-based coating procedures and possibly arises due to increased exciton delocalisation [29] [157].
3.3.4 Peptide conjugation to quantum Dots

Earlier studies have suggested that the surface of MPA-capped QDs can be further derivitised with biological molecules, such as amine-terminated ssDNA, by standard EDC/NHS coupling [37]. It is questionable that amide formation is the exclusive associative mechanism of bioconjugate formation as incubation of MPA-capped QDs with FRET acceptor-labeled DNA, in the absence of coupling agents, results in rapid QD-dye energy transfer, suggesting an appreciable degree of non-specific DNA/QD binding [158]. Furthermore, EDC/NHS has limited capacity for site-selective coupling and renders biomolecules with both carboxy and amine groups susceptible to excessive cross-linking and precipitation [123]. In comparison, self-assembly driven conjugate formation with the absence of covalent bond formation provides a straightforward alternative for the formation of well-defined nanoscale architectures [159] [160]. The peptides employed here were designed to contain an appending hexahistidine sequence (His) which has been demonstrated to self-assemble upon the surface of DHLA-coated ZnS surfaces of QDs [161]. In order to determine a strategy to expedite peptide conjugation to QDs, initial experiments were carried out to determine whether the native surfactant coating of the QD could be replaced.
directly with His6 peptide using a 9:1 (v:v) pyridine:DMSO co-solvent system with a 500-fold molar excess of peptide, predissolved in DMSO. This approach has been demonstrated in the case of replacing TOPO (trioctylphosphine oxide) with cysteine-rich phytochelatin-related peptides on QD surfaces [162] [163]. Here, however, this resulted in QD precipitates which could not be resuspended in aqueous buffers (e.g. PBS, borate) suggesting that peptides were unable to sufficiently replace the native hydrophobic coating under these conditions. Thus, a two-step procedure was employed, involving initial phase-transfer of the QDs to aqueous solution followed by peptide modification. Whilst this approach is slightly more time consuming, it can provide a means to control peptide loading density on the surface of the QD and results in less ambiguity in the final peptide:QD stoichiometry[3].

3.3.4.1 Peptide conjugation to MPA-capped QDs: preliminary Studies

Peptides with sequences based on known substrates for the protein kinases Src and Abl were used in this study. In addition, peptides analogues with tyrosine/phenylalanine substitutions were also synthesised for negative controls in enzyme assays. Initial studies with Src substrate sequences indicated that incubating QDs (100 nM) in 10 mM borate buffer (pH 9.0) with a large excess of peptide (500-fold) would lead to immediate formation of nanoparticle precipitates which were insoluble in aqueous buffer. In case of the possibility of the resultant peptide-conjugate having inherently reduced solubility in aqueous buffer, DMSO (10% v/v) was included in the solution, however, this also lead to nanoparticle aggregation. Therefore, the molar ratio of peptide to QD was systematically varied from 0-100:1 in 10-fold increments to investigate its effect on QD colloidal stability. Similarly, at all ratios examined, all failed to produce stable QD dispersions. Addition of other additives and non-ionic surfactants, such as polyethylene glycol and Tween-20, to colloidal nanoparticle systems has been shown to enhance stability during some ligand-exchange reactions by providing additional steric stabilisation and slowing the kinetics of incoming ligand binding [124][164][165]. Pre-incubation with 0.1% Tween-20 (v/v) for 30 min prior to peptide addition also failed to produce stable QD dispersions. In light of the possibility that the monovalent nature of the MPA thiol-ZnS interaction could influence the absorption kinetics of the peptide and stability of the system, the MPA-QDs were further derivatised with the divalent thiol, DHLA [149]. A 1000-fold excess of DHLA (1 mM in 10 mM borate, pH 9.0)
was incubated with MPA-QDs for 24 hr at room temperature in the dark, followed by removal of excess ligand by repeated ultrafiltration. The DHLA-capped QDs also did not provide stable dispersions following peptide incubation under the same conditions as the MPA-QDs, providing support that the thiol-ZnS interaction was not an underlying factor in determining the stability of the peptide-conjugate.

3.3.4.2 Effect of pH on conjugate stability

The Src substrate sequences used in these studies contained three tandem lysine residues. At pH 9.0, these residues are likely to be in their ionized form (i.e. $\text{NH}_3^+$) and the peptide will have appreciable cationic character. As cationic polyelectrolytes and peptides can cause flocculation of electrostatically-stabilised anionic nanoparticles through charge-screening [11][166], the effect of pH on conjugate formation was systemically studied as it is likely to affect the charge of the peptide [80]. Aliquots of 10 $\mu$M peptide solution were added to MPA-QDs in 10 mM borate buffer at a range of pH values from 9.0 - 11.0 in polypropylene centrifuge tubes. After incubation for 1 hr at room temperature, the samples were centrifuged at 16,000 g for 5 min to indicate if QD flocculation had occurred, as indicated by QD pelleting visible by eye. The photoluminescence intensity in the samples was equivalent across the pH range, suggesting that the QD structure had not been compromised by the pH. These experiments did not reveal any QD pelleting at pH values $\geq 9.6$ suggesting that either the peptide had stably associated with the QD surface or that the elevated pH somehow inhibited the assembly of the conjugate. Thus, gel electrophoresis was employed to further characterise the peptide-QD assembly process.

3.3.5 Agarose gel electrophoresis of peptide-QD conjugates

Agarose gel electrophoresis is a commonly-used analytical technique used in molecular biology to separate biological macromolecules based on size and charge. Nanoparticles can also be subjected to gel electrophoresis to provide low-resolution information on changes in hydrodynamic radius and charge in response to surface modification [167][168][169]. Whilst electrophoresis cannot provide precise molecular information on the nature of the nanoparticle surface ligands, it is a rapid and convenient technique with modest sample requirements. Src substrate peptides were incubated with MPA-QDs at molar ratios from 0-50:1 at pH 9.6 for 30 min prior to loading
into 1 % (w/v) agarose gels. Under 365 nm illumination, concentration-dependent changes in
electrophoretic mobility were observed as a function of peptide:QD ratio, with the mobility of
the QDs being retarded in the presence of peptide compared to MPA-QDs alone, consistent
with peptide assembly on the surface of the QD. Some saturation behaviour was exhibited,
with no further changes in conjugate mobility at peptide:QD > 30:1, suggesting saturation
of potential peptide binding sites (Figure 3.8). This behaviour was also recapitulated in the
case of the peptide substrate and substrate analogue for Abl, suggesting a similar peptide: QD
stoichiometry. This is consistent with other measurements of peptide:QD ratios in the case of
DHLA-capped nanocrystals which suggested much lower experimental loading densities than
those predicted by theory [170]. This was attributed to the existence of a finite number of His₆
binding sites on the QD surface as opposed to a continuous interface to which the peptides can
form a homogeneous closely-packed monolayer. The fact that the two QD-peptide conjugates
exhibit similar electrophoretic mobilities, despite the fact that they theoretically should have
different overall charges (pIs of 10.4 and 6.0 for the Src and Abl substrates respectively) may be
attributed to masking of charged groups by counterions (EDTA, borate etc) in solution, leading
to both of the peptide-QD conjugates having similar net charges.

Dynamic light scattering was also employed to determine changes in the hydrodynamic ra-
dius of the QD following peptide conjugation however, due to limiting amounts of experimental
material, the observed scattering count rates were too low for reliable cumulants fitting (not
shown). Time-resolved photoluminescence experiments, however, provided some additional ev-
dence that peptide binding was occurring on the nanocrystal surface due to observations of
changes in the QD excited state lifetime upon exposure to hexahistidine-appended biomolecules
(_vide infra_). [171].

### 3.3.6 Time-resolved photoluminescence

Electron-hole pair recombination rates have been shown to be highly sensitive to QD surface
chemistry in some instances due to the relatively high surface area to volume ratio associated
with nanoscale materials [66] [172]. Thus, the effect of the peptide coating on QD radiative
lifetime was therefore investigated by nanosecond TCSPC. The water soluble biomolecule-coated
nanocrystals exhibited modified decay profiles (see below for fitted lifetime data), relative to
hydrophobic QDs in chloroform and MPA-capped QDs in aqueous solvent. There are several plausible explanations for this observation; firstly whilst the ZnS shell surrounding the CdSe core helps in the exciton confinement, imperfections in the shell and the surfactant coating play an additional role in passivating the QD. Replacing the native surfactant coating with thiols may increase the non-radiative recombination rate by acting as additional hole-traps [37]. Alternatively, the differences in decay rate may be a reflection of differences in solvent environment, with water molecules providing an additional decay pathways. Addition of peptides and GB1 (30 per QD) further influenced the decay kinetics relative to QD-MPA, increasing the slow component of the decay (Table 3.2). Furthermore, addition of BSA (0.01% w/v) which can assemble on QD surfaces, presumably by electrostatic interaction, to QD-MPA dramatically altered the decay profile [173, 99]. These results support the hypothesis that the peptides used in this study can assemble on the surface of the QDs and tentatively suggest that the His<sub>6</sub> peptides and BSA may be acting to suppress additional decay pathways by either passivating surface trap sites or by providing a protective shield against solvent interactions with the QD surface. Changes in the lifetime of red-emitting (605 nm emission maximum) QDs in basic aqueous solution has been measured as a function of hydrophilic ligand coating, using a range of thiol stabilisers, such as mercaptoacetic acid (MAA), mercaptosuccinic acid (MSA) and dihydrolipoic acid (DHLA) [174]. These experiments revealed only modest differences in lifetime between the
QDs with different ligand-coatings (< 1 ns) compared to the observations here. Surprisingly, there are scant references in the literature to how biomolecule coating can influence the QD excited state lifetime, and these preliminary studies should warrant further investigation into how peptides and proteins of different sequence and/or charge can effect the photoluminescent properties of QDs.

![Figure 3.9: Time-resolved photoluminescence decay profiles water soluble QDs with various biological surface coatings (c.a. 20 nM QD-biomolecule, 10mM borate buffer pH 9.0).](image)

<table>
<thead>
<tr>
<th>Coating</th>
<th>$\bar{\tau}$ (ns)</th>
<th>$\tau_1$ (ns)</th>
<th>$\alpha_1$ (%)</th>
<th>$\tau_2$ (ns)</th>
<th>$\alpha_2$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TOPO (in CHCl₃)</td>
<td>11.8</td>
<td>3.0</td>
<td>43</td>
<td>13</td>
<td>57</td>
</tr>
<tr>
<td>MPA</td>
<td>14.2</td>
<td>2.3</td>
<td>52</td>
<td>16.1</td>
<td>48</td>
</tr>
<tr>
<td>Abl</td>
<td>16.9</td>
<td>2.8</td>
<td>43</td>
<td>18.6</td>
<td>57</td>
</tr>
<tr>
<td>AblF</td>
<td>17.2</td>
<td>2.8</td>
<td>42</td>
<td>18.8</td>
<td>58</td>
</tr>
<tr>
<td>Src</td>
<td>16.2</td>
<td>2.9</td>
<td>47</td>
<td>18.0</td>
<td>53</td>
</tr>
<tr>
<td>SrcF</td>
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<td>2.9</td>
<td>51</td>
<td>17.7</td>
<td>49</td>
</tr>
<tr>
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<td>3.2</td>
<td>36</td>
<td>20.1</td>
<td>64</td>
</tr>
<tr>
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<td>3.2</td>
<td>41</td>
<td>16.5</td>
<td>59</td>
</tr>
</tbody>
</table>

Table 3.2: Average photoluminescence lifetime ($\bar{\tau}$), separate lifetime components ($\tau_1$, $\tau_2$) and relative amplitudes ($\alpha$) after fitting data to a biexponential decay function.

3.3.7 Confirmation of peptide-QD bioactivity by cell imaging

In order to provide evidence that the QD-peptide conjugates retain biological activity to provide useful biosensing reagents, a straightforward and rapid binding assay was developed. To
this end, the QD-GB1 conjugates were allowed to bind to anti-phosphotyrosine or anti-tubulin antibodies and used in a simple nitrocellulose blotting assay or in vitro imaging experiment using phosphotyrosine-BSA and formaldehyde-fixed cells respectively as model target proteins. Using a self-assembly strategy as a means to prepare the antibody-QD conjugate is straightforward compared to the use of other commonly heterobifunctional covalent crosslinkers and ensures that the antibody binding site is not compromised during the QD labeling procedure [175] [147]. This approach is analogous to the use of a similar engineered antibody-binding adaptor protein which assembles on QD surfaces through simple electrostatic interactions [176]. The use of hexahistidine-based self-assembly, however, ensures that the QD-GB1 interaction here is less sensitive to extremes of pH and/or ionic strength. These experiments revealed selective binding to phosphoproteins or tubulin respectively, as revealed by UV-illumination and fluorescence microscopy respectively, providing a simple control experiment and strong support that the MPA-capped QDs can be conjugated to biomolecules by metal-affinity self-assembly without loss of biological function (Figure 3.10). The subsequent goal was to demonstrate that the additional peptide-QD conjugates prepared here can be applied to the detection of protein kinase activity and is detailed in the following chapter.
3.4 Conclusions and Future Work

This goal of this work was to successfully conjugate peptides to hydrophilic quantum dots, with view to developing key components of a novel protein kinase biosensing assay. A range of protein kinase peptide substrates and substrate analogues were successfully conjugated to water soluble MPA-capped QDs by means of a straightforward self-assembly procedure. This yielded QD-peptide conjugates with stoichiometries of c.a. 30:1 as suggested by agarose gel electrophoresis. The QD photoluminescence emission characteristics were shown to be sensitive to the pep-
tide/protein environment as revealed by TCSPC, presumably due to passivation of defect sites on the nanocrystal surface [29]. The biological activity of the QD-peptide conjugates was validated in principle using a simple heterogeneous immunoassay and through cell imaging. These immunosensing reagents may provide a basis for further development of nanoparticle-based imaging studies and form part of an ongoing collaboration with the Lawrence Berkeley National Laboratory. This is the first demonstration that commercially available quantum dots can be rendered water soluble using mild benchtop manipulations and conjugated to biomolecules using a simple self-assembly strategy. This should provide a basis for the rapid development of other biomolecule-QD systems. It was not possible to determine useful additional parameters concerning the peptide:QD interaction (dissociation constant, precise peptide: QD stoichiometry etc), however, future studies could be focussed on addressing these issues. For example, it may be possible to label the peptides with a FRET acceptor fluorophore, titrate the labeled peptide against a fixed concentration of QDs and measure FRET efficiency as a function of peptide:QD ratio. Occupation of all available peptide binding sites on the QD surface should result in eventual saturation of FRET efficiency as a function of peptide:QD; the point at which this is reached should reflect an approximate value for maximum possible peptide coverage [177].

Building from this work, it was sought to apply these new QD-substrate peptide hybrid bionanomaterials to the detection of protein kinases and other important enzyme systems, as detailed in Chapter 5 and 6.
Chapter 4

Protein Kinase Actuated FRET in Quantum Dot - Peptide Bioconjugates

4.1 Background and Introduction

Having successfully demonstrated that peptides and MPA-capped QDs can form stable self-assembling conjugates, it was sought to apply these hybrid nanomaterials to the detection of protein kinase activity. The aim of this study was to demonstrate that QDs can be applied in simple homogeneous FRET-based immunoassays for the detection of enzyme-mediated post-translational modifications.

4.1.1 FRET-based QD immunoassays

There have been several demonstrations that quantum dots can be used in simple homogeneous immunoassays using FRET-based detection. For example, various small-molecule haptens, such as biotin and cortisol, have been conjugated to polymer-coated QDs which can subsequently be recognised by dye-labeled antibodies resulting in QD-dye energy transfer. Such a system can be employed in competitive binding assays [46]. This is a simple procedure but is limited by the
use of bulky polymer stabilisers and the fact that competitive immunoassays are often difficult to optimise [178]. Nonetheless, it was possible obtain very large Förster radii (up to 8.4 nm) with the QD-dye pairs used in this system, thus having great potential for long-range FRET. Other competitive immunoassays have been reported based on the displacement of an acceptor dye-labeled analyte analogue from a recombinant single chain antibody fragment bound to a QD surface. Such a system has been applied to the detection of the explosive 2,4,6-trinitrotoluene (TNT), which removes the quencher-conjugated analogue from the antibody binding site resulting in enhanced photoluminescence emission from the QD [179]. In order to achieve efficient FRET, it was argued that it was necessary to engineer such an antibody fragment to afford short interfluorophore distances. This, however, requires significant development times to obtain an appropriate antibody fragment [180]. Energy transfer between immunoassembled BSA-coated and anti-BSA-coated CdTe QDs with different emission wavelengths has also been suggested, however inter-QD FRET is often problematic due to inevitable direct excitation of FRET acceptor QDs due to substantial overlap in both the donor and acceptor QD excitation spectra and time-resolved measurements are essential to unequivocally demonstrate the extent of such a FRET process [55] [181]. Whilst these are useful and sensitive detection systems, it is notable that none of the reported FRET-based immuno detection methods have so far been developed to detect dynamic biological stimuli, such as enzyme activity, but rather the static presence of an analyte. Here, QDs are engineered to serve as FRET donors in an enzyme-responsive manner.

4.1.2 Experimental design

In considering the ubiquity of immunological-based detection methods in biology, medicine and in vitro assays, it was sought to develop a new simple and generic immuno-FRET procedure for the detection of protein kinase activity. It was predicted that the photoluminescence properties of QDs should render them ideal labels in FRET-based enzyme sensing due to the straightforward ability to tune the QD emission to any spectral region, potentially from the UV to IR, as to suit the user’s end application. This, taken with the additional ability to efficiently excite QDs at any wavelength below their emission maximum, should provide a straightforward means to eliminate potential autofluorescence interference.

Two new approaches were developed which were predicted to obviate the need for polymer-
coated QDs and/or sophisticated recombinant antibody fragments. To this end, water soluble QDs were conjugated to peptide substrates for the tyrosine kinases Src and Abl by metal-affinity mediated self-assembly [182] [62]. These conjugates were then treated with their respective tyrosine kinase in the presence of ATP, leading to enzymatic phosphorylation of tyrosine residues. This was subsequently detected by means of a complementary FRET acceptor-labeled full-length anti-phosphotyrosine antibody or an antibody fragment site-selectively labeled using chemoselective thiol-maleimide coupling (Figure 4.1) [183]. The resultant formation of a diffusing QD-antibody immunocomplex was predicted to lead to substantially decreased spatial separation between the QD and FRET-acceptor dyes and result in efficient QD-dye energy transfer. These two complementary approaches, based on full-length antibodies or antibody fragments, were each predicted to offer several unique benefits. For example, dye labeling of full-length antibodies using amine-reactive probes is a straightforward procedure and can yield high dye:antibody loading densities [184]. Alternatively, antibody fragments (Fab’ fragments), consisting solely of the antigen binding region, are of substantially diminished molecular weight relative to full-length antibodies and thus may have improved steric accessibility to target antigens [185]. Furthermore, the use of site-selectively labeled antibody fragments affords greater predictability in terms of the structure of the final antibody-dye conjugate, as unique thiol groups within the antibody structure can be specifically addressed using chemoselective modification procedures and the diminished size of the macromolecule should be more conducive to smaller interfluorophore distances and more efficient FRET.
Figure 4.1: Experimental design for protein kinase mediated FRET in peptide-QD immunoassemblies. Dye-labeled antibodies selectively bind to enzyme-mediated post translational modification sites, resulting in QD-dye energy transfer (FRET).

4.2 Materials and Methods

4.2.1 Preparation of QD-peptide conjugates

Purified substrate peptides and Y/F substitution controls were incubated with MPA-capped quantum dots (prepared according to materials and methods described in Chapter 3), for 30 min at room temperature (30:1 peptide:QD) in 50 mM sodium borate buffer (pH 9.6). An aliquot of a 1 % (w/v) BSA solution in PBS was then added to a final concentration of 0.1 % and the QDs exchanged into assay buffer (25 mM Hapes (pH 7.5), 10 mM MgCl$_2$) using a 10 kDa molecular weight cut-off centrifugal ultrafiltration device (Millipore). The inclusion of BSA between 0.01-0.1 % (w/v) in the medium was necessary to prevent nanoparticle flocculation in the presence of MgCl$_2$, presumably by affording additional steric stabilisation against electrolyte induced aggregation [186].
4.2.2 Antibody Dye labeling

4.2.2.1 Alexa Fluor 647-NHS ester

Monoclonal anti-phosphotyrosine (clone PT-66, Sigma) was labeled with the amine-reactive N-hydroxysuccinimidy (NHS) ester of AlexaFluor 647 (Invitrogen, Carlsbad, CA). The antibody was buffer exchanged into PBS (pH 7.4) by centrifugal ultrafiltration to remove excess azide from the storage solution and resuspended to a final concentration of 1 mg/ml. Stock solutions of dye (∼1300 g/mol, 4 mM) were prepared in anhydrous DMSO and stored at -80°C before use. The antibody was reacted with a 10-fold molar excess of dye for 1 hr at room temperature with a final DMSO concentration of less than 1% (v/v). Following the reaction, excess dye was removed by repeated rounds of concentration/dilution in PBS by centrifugal ultrafiltration until the flow-through no longer exhibited any dye-specific absorption at ca. 650 nm, as determined by UV-Vis absorbance (note: gel filtration using Sephadex® G-25 as size exclusion media could also be used to remove excess dye. Dialysis against PBS using a 10K MWCO regenerate cellulose membrane (Thermo Fisher Scientific, Rockford, IL), however did not afford adequate dye-removal, even after 24 hr). The ratio of dye:antibody was measured using the extinction coefficient of a typical IgG molecule (ε\(_{280}\) = 203,000 M\(^{-1}\)cm\(^{-1}\)) [187] and that of the dye (ε\(_{650}\) = 239,000 M\(^{-1}\)cm\(^{-1}\)) (Manufacturer’s information) and the expression:

\[
[IgG](M) = \frac{[A_{280} - (A_{650} \times 0.03)]}{203,000}
\]

\[
dye : IgG = \frac{A_{650}}{239,000 \times [IgG]}
\]

where 0.03 is a correction factor to take into account dye-specific absorption at 280 nm. The dye-conjugated antibody was stored in PBS (pH 7.4) containing 0.1 % (w/v) BSA at 4°C before use.

4.2.2.2 Alexa Fluor 647-maleimide

F(ab')\(_2\) fragments were prepared by partial proteolytic digestion of the native anti-phosphotyrosine antibody using agarose-immobilised ficin. The agarose-ficin slurry was pre-equilibrated in 0.1 M citrate buffer (pH 6.0) containing 1 mM cysteine and 5 mM EDTA for 30 min at room temper-
ature before use. 50 µl of 2.2 mg/ml antibody was incubated with 2 ml of the enzyme-agarose slurry and incubated overnight at 37°C with gentle agitation provided by an orbital shaker. An aliquot of the crude digest was subjected to non-reducing polyacrylamide gel electrophoresis and the digest fragments visualised by coomassie staining (Imperial Protein Stain, Thermo Fisher Scientific, Rockford, IL) to verify cleavage of the antibody. The digest mixture was purified by incubation with agarose-immobilised protein A to remove Fc fragments of the antibody. The disulphides in the hinge region of the F(\(ab'\))\(_2\) were reduced using 50 mM 2-mercaptoethyamine in the presence of 10 mM EDTA at 37°C for 90 min and the antibody fragments (Fab') purified by centrifugal ultrafiltration. The Fab' reduced thiols were then labeled with Alexa Fluor 647 C\(_2\)-maleimide (20-fold excess) overnight at 4°C and the conjugate again purified by centrifugal ultrafiltration. The ratio of dye:Fab' was calculated by UV-vis absorption, using the Fab' extinction coefficient (\(\epsilon_{280} = 750,000 M^{-1} cm^{-1}\)).

4.2.3 Enzyme reactions

Recombinant GST-purified Src (SignalChem, Richmond, Canada) and Abl (Calbiochem, La Jolla, CA) tyrosine kinases were used throughout this study and stored as single-use aliquots at -80°C. GST-purified enzymes were chosen, as opposed to hexahistidine-tagged enzymes, in order to avoid potential ligand exchange with QD-bound hexahistidine-terminated peptides. Stock solutions of 10 mM ATP (adenosine 5'-triphosphate disodium salt, ≥ 99 % purity, Sigma) were prepared in 10 mM HEPES, pH 7.5 and stored at -80°C before use. Generic kinase reaction buffer consisted of 25 mM HEPES (pH 7.5), 10 mM MgCl\(_2\) containing 0.1 % (w/v) BSA. The buffer was passed through a 0.45 µm syringe filter (Millipore) before use and prepared fresh on the day of experiment. 10 µl dilutions of enzyme were incubated with 10 µl of QD-substrate peptide conjugates (60 nM) and 5 µl 1 mM ATP in polypropylene tubes for 1.5 hr at 30°C. The reactions were quenched by chelating free MgCl\(_2\) with the addition of 20 µl 50 mM EDTA prepared in 25 mM HEPES (pH 7.5) for 10 min at 30°C. A 4-fold molar equivalent of anti-phospho-tyrosine-AF647 (full-length IgG or Fab' fragment) to QD was added to the sample an allowed to incubate for 30 min before recording photoluminescence spectra. The precise volume of anti-phospho-tyrosine-AF647 added was dependent on the concentration of the conjugate, as determined by UV-vis absorbance.
4.2.4 Inhibitor titration

Stock solutions (1 mM) of the broad spectrum protein kinase inhibitor, staurosporine (Calbiochem, La Jolla, CA) were prepared in anhydrous DMSO and stored at -20°C before use. Triplicates of 5 U of Abl (10 µl) and 10 µl of QD-peptide conjugate (30 nM) were incubated with 10 µl of serial dilutions of inhibitor in 25 mM HEPES, 10 mM MgCl₂, 0.1 % (w/v) BSA for 30 min at room temperature in 384-well black microplates (Cornig, Rochester, NY), before initiating the kinase reaction with the addition of 1 µl ATP (final [ATP] = 12.5 µM ≈ K_M ATP). The reactions were incubated for 1 hr at 30°C before quenching the enzyme reaction with EDTA and antibody detection, as described. The photoluminescence and fluorescence emission intensities at 605- and 670 nm were measured on a DTX 800 multimodal plate reader (Beckman Coulter, Fullerton, CA) with 400 nm excitation.

4.3 Results and Discussion

4.3.1 Fluorophore labeling

The use of QDs and acceptor fluorophores with red-shifted emissions (i.e. > 600 nm) facilitates the rejection of biological autofluorescence which tends to occur at shorter wavelengths. In addition, in drug discovery, autofluorescence originating from test compound libraries is a common source of signal interference in fluorometric assays. The ability of a molecule to absorb and emit longer wavelength (i.e. red) light is largely a function of the degree of its conjugation. This necessary high degree of conjugation is not normally represented to a large extent in typical compound libraries, therefore red-emitting dyes show notable advantages as a means to reduce signal artefacts due to autofluorescence [20]. Thus, considering their emission profile, the QD605-AF647 pair was determined to be a particularly suitable candidate for FRET sensing (Figure 4.2).
Figure 4.2: Normalised absorption (dashed line) and emission (solid line) spectra of QD605 (blue) and Alexa Fluor 647 (red)

This FRET pair also exhibits a large Förster distance of 71.5 Å, comparable to the exceptionally large distances observed with lanthanide-based donors (65-90Å [42]), rendering it particularly suitable for energy transfer in large macromolecular assemblies [46][42]. As the dimensions of typical IgG molecules (14.5 x 8.5 x 4.0 nm) are reasonably large with respect to high-efficiency FRET regimes associated with typical organic dyes, the QD605-AF647 pair was predicted to be conducive to efficient E [119][188]) .

Two antibody labeling procedures were investigated. In the first approach, the antibodies’ free amines were randomly targeted with an NHS ester-AF647 derivative (Figure 4.3), typically providing an IgG:dye stoichiometry of 4-5:1 as determined by UV-vis absorbance. This degree of labeling was decided upon based on maximising the number of FRET acceptors per IgG whilst minimising overlabeling which could result in dye self-quenching and/or compromising the antibody’s antigen binding site [189]. Alternatively, in order to afford dye:antibody conjugates of better defined structure and stoichiometry, the antibody’s Fv antigen binding fragment was site-selectively labeled using a thiol-reactive maleimide-derivative of AF647 (Figure 4.4), following partial enzymatic digestion of the native IgG and mild reduction of the hinge-region disulphides to expose free thiols for chemoselective fluorophore conjugation. Antibody fragment generation and purification was confirmed by SDS-PAGE and protein staining, revealing a single 100,000 KDa band after protein A purification, corresponding to F(ab’)2 (Figure 4.5). The final yield
of the Fab'-AF647 fragment was 30\%, as determined by UV/vis absorption.

Figure 4.3: NHS-AF647 labeling of IgG amines

Figure 4.4: Chemospecific maleimide-thiol labeling of Fab' thiols
4.3.2 Protein kinase activity assays: steady state photoluminescence

Following addition of AF647-antiphosphotyrosine antibody to the kinase reaction mixtures, containing ATP, enzyme, peptide-QD conjugate and EDTA, a concomitant increase and decrease in photoluminescence emission at 670 nm and 605 nm respectively as a function of enzyme concentration was observed for both Src and Abl. Furthermore, this behaviour was an ATP-dependent process and was not observed in the case of QD-conjugates which employed non-phosphorylatable Y/F substitutions in the substrate sequence, consistent with kinase-activity dependent energy transfer (Figure 4.6). Under the experimental conditions here, the maximum observed FRET efficiencies, \( E \), where \( E = 1 - \frac{F_{DA}}{F_D} \), were 76 % and 45 % for Abl and Src respectively. Considering the similar size of the two substrates (18 and 15mer peptides), it is unlikely that the observed differences in FRET efficiency are due to differences in peptide structure and overall D-A separation. However, it is possible that the two peptides may have slightly different assembly mechanisms and/or packing structure on the QD surface and may
Figure 4.6: Steady-state photoluminescence spectra following addition of FRET-acceptor antitriphosphotryosine. (A) Abl and (B) Src concentration-dependent FRET behaviour; ratiometric limit of detection for Abl (C) and Src (D)

not necessarily have equivalent numbers of enzyme-accessible tyrosine residues, thus providing a limit to the maximum-observed FRET efficiency. For example, the KKK motif, in addition to the appending hexahistidine, in the Src substrate peptide may also influence the orientation of the peptide on the QD surface as primary amines have some affinity for ZnS [190]. Alternatively, the observed discrepancies may reflect subtle differences in enzyme-substrate preference and/or specific assay buffer requirements for maximum enzyme activity.

Plotting the emission intensity ratios (670 nm/605 nm) against enzyme concentration was carried out to determine approximate limits of detection. A reaction mixture which omitted ATP was chosen as a baseline control. This revealed detection of 0.33 nM (25 mU/µl) Abl and 2.3 nM (11.25 mU/µl) Src (signal:background = 4). This sensitivity is commensurate with other widely used kinase assays and other nanoparticle-enable detection technologies [191, 192, 193, 11, 194]. In addition, this method has significant advantages over other nanoparticle-based methods due to the fact that only a single biofunctional nanoparticle is required, as opposed to a heterogeneous mixture of nanoparticles with different biological coatings to provide both enzyme-responsive and signal-transducing components. It was demonstrated here that by
simply changing the peptide substrate sequence, different kinases could be readily detected without need of substantial redesign of the system. This suggests that the system here is flexible and may provide a universal assay platform to detect the activity of a range of unrelated kinases. Furthermore, self-assembly of the conjugates also affords greater simplicity in terms of preparation and purification. Finally, it should be possible to enhance the sensitivity of this kinase detection system by means of the use of antibodies with higher dye loadings, thereby increasing the ratio of acceptor to donor, or the use of dye-QD pairs with larger Förster radii, thus increasing energy transfer efficiency.

4.3.3 Protein kinase activity assays: time-resolved photoluminescence

To further verify that enzyme activity was actuating energy transfer, the QD lifetime in the presence of different concentrations of kinase was investigated using TCSPC measurements (Figure 4.7). QD-dye FRET should be manifested as a decrease in QD excited state lifetime. These experiments revealed a decrease in average lifetime as a function of enzyme concentration, in an ATP-dependent manner, consistent with the model of specific protein kinase activity actuated FRET.

Figure 4.7: Time resolved photoluminescence emission traces of Abl reactions in presence of FRET-acceptor labeled antibody. Average lifetimes, $\tau$, were obtained from fitting the data to a biexponential decay.

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Figure 4.8: Time resolved photoluminescence emission traces of Src reactions in presence of FRET-acceptor labeled antibody. Average lifetimes, $\tau$, were obtained from fitting the data to a biexponential decay.

4.3.4 Optimisation of antibody:QD ratio

The effect of antibody concentration on FRET efficiency was investigated to ascertain whether there was an optimum antibody:QD ratio and in order to avoid utilising the antibody in excess to improve overall cost-effectiveness. Therefore phosphorylated QD reaction products (200 mU/µl Abl) were incubated with different amounts of antibody to achieve different final antibody:dye ratios, the photoluminescence spectra recorded and the 670 nm/605 nm emission ratio recorded. Saturation behaviour was observed at ratios $> c.a \, 4:1$ (Figure 4.9). This possibly reflects steric crowding effects on the QD surface as only a finite number of antibodies ($14.5 \times 8.5 \times 4.0 \, \text{nm}$) will be able to pack around the central QD (c.a. $4 \times 9.4 \, \text{nm}$, manufacturer’s information), however, given the limited information on the precise structural details of the QD-peptide conjugate, it is difficult to quantitatively model the QD-antibody interaction and its implications to FRET.
Figure 4.9: Effect of antibody:QD on energy transfer; (left) Steady state photoluminescence spectra; (right) 670 nm/605 nm emission intensity ratio. The asymptote reached at ratios >4:1 antibody:QD suggests saturation of the QD surface, providing an upper limit to the number of antibodies per QD required to elicit the maximum signal response.

4.3.5 Gel electrophoresis of kinase reaction products

Enzymatic phosphorylation of the QD peptide coating should lead to a change in surface potential due to the increased negative charge imparted by the phosphate group. It was predicted that the electrophoretic mobility of the QD should change following phosphorylation and that this could be verified by agarose gel electrophoresis [13]. Abl reaction mixtures (200 mU/µl) with inclusion or omission of ATP and/or the antiphosphotyrosine antibody were run on agarose gels. From these observations, there was no discernable difference in electrophoretic mobility between reactions which included or omitted ATP. This may be due to BSA binding to the surface of the QDs and masking of any subtle electrochemical effects that may influence overall electrophoretic mobility. The addition of the antiphosphotyrosine antibody was predicted to result in a retardation in electrophoretic mobility of the peptide-coated QDs owing to the substantially increased size of the resultant immunocomplex, provided that the immunorecognition is not compromised within the gel matrix in the presence of an applied external electrical field. Some band broadening was observed in this case, tentatively suggesting that there may be some heterogeneity in the QD-antibody interaction e.g. different antibody:QD stoichiometries, resulting in a range of QD-antibody complexes with different electrophoretic mobilities.
4.3.6 Measure of Assay Robustness (Z’-factor)

In order to determine whether the assay could have potential as a high-throughput screening tool, a quantitative measure of assay reliability was sought. One such parameter used to validate the quality of high-throughput screens is the Z’-factor. This is a dimensionless coefficient which reflects the assay signal’s dynamic range, data variation and reliability in identifying statistically significant hits from a compound library screen [195]. It is defined using the means, μ, and standard deviations, σ, of the positive (p) and negative (n) controls:

$$Z' = 1 - \frac{3 \times (\sigma_p - \sigma_n)}{|\mu_p - \mu_n|}$$

Thus, Z’ takes values between 0 and 1, with assays with Z’ = 1 being ‘perfect’ and Z’ values
> 0.5 being considered to have excellent potential in HTS. The applicability of the QD kinase assay to HTS was determined by carrying out Abl reactions, as described, at fixed concentration (200 mU/µl) for positive and negative (phenylalanine/tyrosine substitution) controls ($n = 10$) and recording the photoluminescence spectra. The intensities at 605 and 670 nm were converted to a ratio $670/605$ and the means and standard deviations calculated (Figure 4.11, Figure 4.12). This provided $Z'$ values of 0.75, suggesting that the assay may be suitable for HTS applications. Equipment limitations prevented larger scale analysis of $Z'$ in a genuine high-throughput context, however, automated liquid handling systems capable of dispensing many hundreds of experimental replicates into microplates should provide even more confident $Z'$ values to further validate this assay as a drug-discovery tool.

Figure 4.11: Determination of $Z'$-factor from PL spectra of Abl kinase QD reactions; Y/F negative control (left); positive control (right), 200 mU/µl Abl
Figure 4.12: 670/605 nm emission intensity ratios derived from steady-state photoluminescence data used to calculate Z'-factor. 10 independent experiments were conducted which employed either the Abl cognate substrate (black squares) or the non-phosphorylatable phenylalanine substitution (red circles) conjugated to the QD FRET donor.

4.3.7 Staurosporine Inhibition

As the assay appeared to show potential in HTS, further tests were carried out to investigate whether it could be applied to quantitatively detect enzyme inhibition. To this end, the broad spectrum kinase inhibitor, staurosporine, was incubated with Abl prior to initiating the kinase reaction with ATP [196]. Inhibitor compounds libraries in HTS are typically pre-dissolved in DMSO, with a final solvent concentration of 0.5-1.5 % and, furthermore, Staurosporine itself is only sparingly soluble in aqueous solution [197]. Thus it was necessary to test the tolerance of the QD-peptide conjugates to DMSO at a commensurate concentration. At concentrations of at up to at least 25 % DMSO, there were no observable changes in either colloidal stability or photoluminescence properties of the peptide-QD conjugates, suggesting that the system was compatible with the use of low concentrations of polar organic solvents.
Staurosporine is a microbial alkaloid which acts as a competitive ATP binding-site inhibitor (Figure 4.13) [198]. As such, it is necessary to conduct assays at subsaturating concentrations of ATP, typically within an order of magnitude of the $K_M$. Preliminary studies were thus carried out to find appropriate concentration range in which to test the sensitivity of the assay to Staurosporine by performing kinase reactions in the presence of different amounts of ATP. This provided an apparent $K_M$ value of 12.0 µM; consistent with previously reported literature values of 12-18 µM [111]. Kinase reactions were then carried out in the presence of different concentrations of staurosporine and phosphorylated QDs detected as described. Plotting the 670/605 nm ratio against [staurosporine] revealed dose-dependent sigmoidal behaviour, as predicted for the case of specific enzyme inhibition. This provided a staurosporine IC$_{50}$ value (i.e. half maximal inhibitory concentration) of 100 nM; comparable to previously reported literature values of 76 nM generated using a fluorogenic peptide / protease-coupled kinase assay [111]. This provides strong evidence that the assay developed here may have utility in the quantitative determination of potential inhibitor potency and may serve as a drug discovery tool.
4.3.8 Investigation of other assay formats

It is possible to consider a number of potential assay designs, with variations in the arrangement of peptides, antibody, QDs and dye. One of the fundamental design criteria is that QD-dye distances should be minimised as to favour efficient energy transfer. To this end, it was sought to develop an alternative to the use of relatively large-sized native IgG which would allow smaller interfluorophore distances to be accessible. The use of site-selectively labeled Fab’ (i.e. antigen binding) fragments was predicted to be a suitable alternative phosphotyrosine recognition moiety and offer several advantages compared to the use of full length IgG molecules. For instance, more robust theoretical predictions of interfluorophore distances can be made owing to the unambiguous site of dye-labeling afforded by chemoselective thiol-maleimide conjugation and greatly simplifies FRET data interpretation. In this case, the system could be treated as centrosymmetric with dyes equidistantly arranged around a central QD donor, with the FRET equation reducing to:

\[ E = \frac{nR_0}{nR_0 + r^6} \]

where \( n \) is the number of acceptors surrounding the donor [3]. In addition, the lower molecular weight of the truncated antibody conjugate (7 nm × 5 nm × 4 nm) could afford higher dye:QD stoichiometries owing to enhanced steric accessibility compared to the larger full length antibody.
Figure 4.15: Time-resolved photoluminescence decays of Abl kinase reactions (200 mU/µl) using Alexa Fluor 647 maleimide labeled antibody Fab’ fragments (c.a. 50:1 Fab’:QD).

F(ab’)2 were generated by partial enzymatic digestion with Ficin - a cysteine protease derived from fig latex - which preferentially cleaves the Fc hinge region and the thiols reduced and modified with the maleimide derivative of Alexa Fluor 647. These fragments were then utilised in QD kinase activity assays for Abl (500 mU/µl) in an analogous manner to the full-length IgG, and the time-resolved photoluminescence decays were recorded to detect FRET. The decay times in the absence and presence of Fab’ or where a non-phosphorylatable Y/F substitution peptide was used did not provide any evidence for changes in donor lifetime suggesting that FRET was not an active process. Indeed, the observed decays were superimposable, giving average $\tau$ of 16.8 ± 0.8 ns (Figure 4.15). This could be due to the antibody binding site being compromised following the dye labeling procedure due to the extended period spent at elevated temperature during proteolytic generation of the Fab’ fragment. Owing to limiting amounts of material and with the possibility that the approach may be intrinsically unfeasible, the use of Fab’ fragments as immunodetection reagents in the assay was not investigated further.

Whilst it may be possible to produce similar fluorophore-antibody conjugates of well-defined structure, it should be emphasised that enzymatic means of preparation involve extensive processing, purification and have limited throughput. In summary, although there are benefits in
having reduced ambiguity in the conjugate structure, this must be weighed against potential limitations associated with relative ease of conjugate preparation. For example, it should be possible to site-selectively label the Fc region of the phosphotyrosine antibodies by targeting carbohydrate moieties with hydrazide-modified acceptor dyes [124]. Although this should not compromise the antigen binding site the integrity of the antibody’s antigen binding site, this would presumably result in the FRET acceptor dyes being oriented relatively far from the QD donor and may preclude efficient energy transfer.

4.4 Conclusions and Further Work

Quantum dots coated with judiciously designed peptide sequences can serve as surrogate substrates in a new protein kinase activity assay based on immunorecognition-actuated FRET and can be applied to screening potential enzyme inhibitors. The assay is simple and does not require the need for sophisticated instrumentation. In principle, the luminescent readout of the system could be readily tailored by simply changing the D-A FRET pair to suit the user - for example, NIR emitting QDs/dyes should further provide a means to limit autofluorescence interference. The strong distance dependence of FRET prevents unbound antiphosphotyrosine antibodies from contributing to the measured signal and affords convenient homogeneous reactions. Whilst the dimensions of most antibodies are large relative to distances associated with high-efficiency FRET regimes [145], the efficient FRET (76%) observed here can be attributed to several design features. Namely, the small-hydrodynamic radius of the peptide-QD conjugate afforded by self-assembly assists in minimising D-A distance separation, QD-dye D-A pairs can exhibit relatively large Förster radii [46] and each quantum dot is capable of binding to multiple antibodies, permitting a high overall dye: QD ratio. It may be possible to further increase the maximum FRET efficiency by increasing dye loading on the antibody and investigating other QD-acceptor pairs which exhibit larger Förster radii [48]. This could facilitate the use of larger full-length His6-appended recombinant protein substrates, as opposed to synthetic peptides, as although they would more accurately mimic endogenous protein substrates, their larger relative size would result in an increase in the distance of closest approach between the QD donor and dye acceptors. In addition, the antibody dye-label could be substitute for a lanthanide cryptate to allow the QD to serve as an energy transfer acceptor as opposed to
donor to enable time-resolved measurements and further assist in temporal discrimination of background signal [51]. This approach should also provide a convenient means of harnessing the multiplexing capabilities of QDs. This would be of particular use in the context of discovery of selective kinase inhibitors and would provide a means to assay two or more enzymes simultaneously in an individual reaction. For example, two or more QDs with well-separated emission wavelengths could be conjugated to distinct substrate peptides for kinases with non-overlapping substrate specificity followed by phosphorylation in the presence of the respective kinases. A generic phosphoamino acid antibody could then be added to the system, itself conjugated to a europium or terbium cryptate, and allowed to bind to the phosphorylated peptides. Subjecting this heterogeneous population to an excitation pulse, followed by a time delay and subsequent measurement of QD photoluminescence should result in the observation long-lifetime energy transfer from the cryptate to the QD, with the respective QD emission intensities corresponding to the relative degree of phosphorylation of the distinct QD-peptide conjugates [69].

In principle, provided the availability of appropriate antibodies, it should be possible to extend the assay principle to other kinases, including serine/threonine kinases [107]. This work is currently being built upon by applying the assay to the detection of other medically relevant kinases, specifically the catalytic domain of the human epidermal growth factor receptor 2 (HER2) - commonly mutated or overexpressed in breast and lung cancer [199] - in order to determine its utility as a potential diagnostic tool in a multiplexed kinase/protease assay.

As kinase assays are often carried out in crude cell lysates in drug discovery applications [200][107], it would be interesting to test the QD-based assay in a similar context to further validate its applicability as a pharmaceutical screening tool. An intriguing potential application of this QD-kinase sensing approach would be its utility in measuring kinase activity within live cells. This would necessitate the development of suitable strategies to deliver QD-peptide substrates and/or antibodies to the cytosol and thus presents a significant challenge. This could be realised using various methods developed for gene transfection based on the use of cationic polymers, cell-penetrating peptides, surfactants, liposomes or electroporation to deliver macromolecule payloads within the cell by providing a mechanism to pass through the plasma membrane or escape endosomal pathways [142]. Nonetheless, successful and stable delivery of both QD and antibody to the cell followed by an enzyme-dependent FRET response would
likely require substantial modifications to the system in consideration of the protein-rich and complicated milieu of the intracellular environment. For instance, the propensity for peptide dissociation from the nanocrystal surface, proteolytic cleavage or non-specific binding may provide sources of complication to intracellular enzyme sensing [201]. An initial proof-of-concept study could involve the optimisation of QD-substrate peptide delivery to kinase (+) and kinase (-) cells, followed by fixation of the cells and immunostaining with a FRET-acceptor labeled anti-phosphoamino acid antibody and FRET-based immunodetection by standard fluorescence microscopy. Although this procedure would not provide a route to live-cell imaging, this experiment would provide valuable insight into whether the QD-peptides have biological activity in an intracellular environment and can be acted upon a cognate kinase in a physiological context.

An important limitation of the assay here is its dependence on the use of potentially expensive antibodies. It may be possible to substitute antibodies with other possible affinity ligands. For instance, it may be possible to engineer suitable aptamers, metal-chelate based affinity ligands or naturally occurring phosphoresidue binding domains (e.g. SH2 domains) to serve as specific phosphorecognition motifs [202]. Another potential antibody-free assay could employ γ-biotin-ATP, resulting in kinase-mediated biotinylation of the QDs, followed by detection with a FRET-acceptor labeled streptavidin [203]. In light of the fact that QD photoluminescence is often critically dependent upon environment and surface ligand chemistry, it may be possible to exploit the appreciable changes in surface charge upon phosphorylation for an antibody-free based approach. For example, for some QD compositions, phosphorylation of the peptide coated-QDs could be manifested as an altered excited-state lifetime, shift in the photoluminescence emission maximum or changes in the emission intermittency statistics (i.e. blinking) [66]. Alternatively, many protein kinases can utilise γ-S-ADP as a surrogate co-substrate to substitute for ATP. The use of γ-S-ADP results in enzymatic incorporation of thiol groups into the target substrate and provides a useful chemical handle for further derivitisation. Such enzymatic chemical modification has been used to incorporate thio-phosphate affinity tags into potential kinase targets, which are subsequently captured by a thiol-reactive probe to allow phosphoprotein enrichment [204]. An analogous approach could be adopted here, whereby the QD-peptide is enzymatically thiolated and subsequently reacted with a chemically-modified FRET reporter dye to afford antibody-free detection.
An additional limitation of the assay lies in reagent preparation, due to the need for QD-peptide solubilisation, preparation and purification. The ability to use reagents from commercial sources could simplify the assay, but would require appreciable redesign. For example, it may be possible to use commercially available streptavidin coated QDs in conjunction with a biotinylated kinase substrate and lanthanide-labeled anti-phosphoamino acid antibody to avoid the need for in-house nanoparticle derivitisation.

Finally, these initial results raise the interesting possibility that QDs may be utilised to detect other enzyme-catalysed modifications distinct from phosphorylation, providing a universal platform to detect other enzyme activities and related post-translational modifications. Chapter 5 details another embodiment of this enzyme-detection strategy, by applying it to measure the activity of an unrelated family of enzyme.
Chapter 5

Quantum Dot Probes for Histone Acetyl Transferase Activity

5.1 Introduction

Given the demonstration that QDs can be tailored to detect the activity of protein kinases, it was sought to investigate whether the approach was generalisable and if it could be applied to detect the activity of unrelated enzymes which mediate post-translational modifications. This chapter describes the development of QD systems designed to measure the activity of histone acetyltransferases (HATs).

HATs catalyse the acetylation of lysine residues of target proteins using acetyl-CoA as an acyl donor co-substrate (Figure 5.1). This has the effect of neutralising the lysine side chain, typically cationic at physiological pH, and creates new opportunities for interaction with acetyl-lysine binding proteins [205]. HATs play a crucial role within the nucleus, where they serve to preserve the integrity of the genome and provide additional levels of transcriptional regulation of gene expression [206, 207]. Although the knowledge of the precise DNA base pair sequence of many genomes has yielded a wealth of powerful evolutionary and functional information concerning gene function, it is becoming increasingly apparent that this is insufficient to assist in our comprehensive understanding of how gene expression is regulated in a tightly controlled temporal and cell-specific manner [208, 209]. This is of great importance to fully understand
fundamental cellular processes such as development and stem cell differentiation. In order to provide such levels of additional regulation, eukaryotic cells employ an elaborate system of chemical modifications which act upon DNA itself and a host of proteins which intimately interact with DNA and have a profound influence on higher-order DNA structure and gene expression. These modifications serve to assist in defining when and to what extent certain genes are expressed. This functional information encoded by such protein- and DNA-chemical modifications is known as epigenetics [210, 211].

The genome of eukaryotic cells is condensed into the nucleus by means of the formation of protein-DNA interactions mediated by histone proteins to form higher-order structure known as chromatin. Histones are highly basic proteins consisting of two copies of the core proteins H2A, H2B, H3 and H4 arranged to form an octameric complex. The individual histone proteins consist of a globular C-terminal core domain and a flexible lysine-rich N-terminus ('tail') which protrudes from the core, as revealed in the X-ray crystal structure [212]. 146 base pairs of DNA are spooled around the nucleosome structure in a left handed superhelix to provide the fundamental repeating unit of chromatin structure - the nucleosome core particle (Figure 5.2) - and additional accessory proteins further facilitate condensation of the DNA. This process results in a $\sim 10^4$-fold compaction which must be reconciled with the fact that a multitude of DNA-binding proteins require unhindered access to their target sequences in order to maintain tight temporal
regulation of fundamental processes associated with DNA metabolism, such as replication, repair, recombination, transcription and integration of extracellular signals with changes in nuclear activity. It is now well understood that underlying chromatin structure plays a fundamental role in the regulation of these processes which, in turn, is determined by precisely controlled post-translational modification of histone side chains. The lysine-rich N-terminal histone tails are subject to a wide range of reversible modifications including acetylation, methylation, phosphorylation, ubiquitinylation, ADP-ribosylation and SUMOylation (Table 5.1) [213, 209, 214]. These modifications serve as recruitment sites for a plethora of transcription factors and other effectors of chromatin structure by means of complementary protein domains which are capable of recognising specific side-chain modifications [215]. The notion that an underlying language exists which relates combinations of histone modifications to specific changes in chromatin structure and gene expression is known as the histone code hypothesis [208].

There is increasing evidence that histone modification and global epigenetic variations may play a significant role in the development of several disease states by means of several potential mechanisms [221]. For example, trimethylation of lysine 9 of histone 3 (H3K9me3) is typically associated with the establishment and maintenance of transcriptionally silent heterochromatin and is essential to preserve genomic integrity. The protein, GASC1, has been found to be over-

Figure 5.2: The nucleosome core particle complexed with DNA (Protein Data Bank code 1AOI) with N-terminal histone tails protruding from assembly & known mammalian histone tail acetylation targets and associated enzymes (adapted from Abcam.com)
Table 5.1: Histone modifications and targeted residues [213] [216] [217] [218] [219] [220]

expressed in oesophageal squamous cell carcinoma and possesses H3K9me3/H3k9me2 demethylation activity, as observed in vitro and in vivo, and aberrant overactivity may contribute to derepression of previously silent oncogenes [222]. In addition, mouse models of Huntington’s disease suggest that aberrant epigenetic control of transcription plays a role in disease etiology, as treatment with histone deacetylase (HDAC) inhibitors results in increased histone acetylation in the brain and correlates with amelioration of disease symptoms [223]. Global analysis of histone acetylation patterns in prostate cancer cells can also serve as an accurate indicator of disease prognosis [221]. In addition to cancer, histone acetyl transferases have also been implicated in chronic inflammation, such as asthma and chronic obstructive pulmonary disease (COPD) [224]. Finally, integration of HIV-1 genetic material into the human genome, catalysed by the viral integrase protein (IN), is potentiated by p300-mediated acetylation of conserved lysine residues within the C-terminal region of IN [225]. Considering the broad disease implications of aberrant HAT activity, HATs are an emerging class of drug target and the ability to measure HAT activity is an obligatory step in the development of new small molecule activity modulators.

5.1.1 HAT assays

Radioisotopic labeling techniques using $[3H]$ acetyl-CoA are one of the most widely used methods used to detect HAT activity owing to the combined benefits of sensitivity and versatility in assay design. For example, they can be applied to study the acetylation of full length histone substrates or synthetic peptides followed by separation of the isotope-labeled substrate by means of gel electrophoresis or filter binding and quantitation by phosphorimage analysis or scintillation counting [226]. Owing to the need for additional separatory steps and complications associated with waste disposal, other colourimetric and fluorometric assays have been developed. For example, it is possible to convert the HS-CoA by-product generated during the course of the
HAT reaction into a colourimetric signal by means of an enzyme coupled reaction generating reduced NADH and concomitant increased absorption at 340 nm or reacting the HS-CoA with maleimide derivatives of coumarin dyes to form a fluorescent adduct[227].

In addition to these approaches, there are some reports which make use of nanoparticle-specific phenomena to examine histone modifications. For example, surface enhanced Raman scattering using silver colloids can be used to detect and discriminate different histone tail modifications with high-sensitivity (zmol) by means of their distinct associated spectral signatures [228]. This approach was limited to the use of highly purified peptides prepared by chemical synthesis and may be unsuitable for analysis of in vitro enzyme reactions, where a relatively heterogeneous sample composition could complicate peak assignment in the SERS spectrum. In addition, quantum dots have been applied to screen on-bead peptide libraries for preferred substrate sequence specificity of histone deacetylases (HDACs) [229]. Here, a library of bead-immobilised peptide sequences, each containing an acetyl-lysine residue flanked by random amino acid residues, was treated with the HDAC SIRT1, leading to the generation of free lysyl ε-amino groups. These groups were subsequently detected by modification with NHS-biotin followed by binding to streptavidin-coated QDs. It is possible to envisage how this approach could be modified to detect HAT activity (i.e. the antagonistic acetylation enzyme reaction) and probe substrate specificity by employing non-acetylated peptides in conjunction with QDs labelled with an appropriate anti-acetyl-lysine antibody or another suitable affinity motif. Nonetheless, the heterogeneous nature of this system limits its applicability to high throughput screening for potential modulators of enzyme activity.

5.1.2 Aims and scope of the chapter

In consideration of the current assay methods, it was sought to adapt the previously described QD-kinase technology to the detection of HAT activity. The catalytic domain of the transcriptional coactivator p300 was chosen as a model system to develop a new assay for HAT activity owing to its comprehensive biochemical and structural characterisation and its clinical significance [230]. It was sought to design and synthesise appropriate peptide substrates for p300, conjugate these peptides to QDs, and apply the resultant peptide-conjugates to detect HAT activity in an analogous manner to the previously developed kinase system. This was chosen
as to demonstrate that despite the fundamental disparities in enzyme structure, function and catalytic mechanism between kinases and HATs, the QD assay principle can be readily adapted to detect the activity of an arbitrary choice of transferase enzyme, providing a potential route to a universal assay format for transferases which could be rapidly tailored to detect different enzymes by simply modifying the peptide substrate sequence and antibody specificity. This chapter details the development of a novel QD-based HAT assay, the initial studies conducted to develop the HAT sensing system, vital control experiments carried out to guide essential redesign of the assay format, assay optimisation, further characterisation and demonstration that QDs can be successfully applied to quantitatively screen for potential inhibitors of HAT activity.

5.2 Materials and Methods

5.2.1 Peptide synthesis

A hexahistidine-terminate peptide substrate for p300 based on the N-terminal tail sequence of the H4 protein ($H_2N\text{RGKGGKGLGKGAHHHHHHH-CONH}_2$) was synthesised by HOBr/HBTU coupling of Fmoc-protected amino acid monomers in NMP using a 10-fold excess of amino acids per coupling step. The peptide was cleaved from the resin, purified, lyophilised and characterised by MALDI as described previously. This peptide sequence was derived from residues 4-12 of the N-terminal tail region of H4 and carries all necessary determinants to serve as a cognate substrate for p300 [231].
5.2.2 Bioconjugate preparation

QDs were rendered water soluble and conjugated to the purified His\textsubscript{6}–appended substrate as described in Chapter 3. Monoclonal anti-acetyl lysine (clone 7F8) was labeled with AlexaFluor 647 - succinimidyl ester as described in Chapter 4.

5.2.3 p300 HAT assay

Stock solutions of the purified recombinant catalytic domain of p300 (Millipore, MA) were diluted in assay buffer (50 mM Tris-HCl (pH 8.0), 10 % (v/v) glycerol, 0.1% (w/v) BSA) and incubated with 20 µM H4-tail peptide and 100 µM acetyl-CoA (trilithium salt, Sigma-Aldrich) in a total volume of 10 µl for 2 hr at 30°C. A 2 µl aliquot of the reaction mixture was added to 10 µl of a 50 nM QD solution in assay buffer to allow peptide self-assembly on the nanocrystal surface. Steady-state photoluminescence spectra were recorded on a Spectramax M5 microplate reader (Molecular Devices) using 400 nm excitation.
5.2.4 Peptide chemical acetylation

Nucleophilic $\epsilon$-amino groups of lysine residues, arginine guanidium groups and peptide N-termini are susceptible to chemical acylation by acetic anhydride [123]. 1 mg of H4-tail peptide was dissolved in 500 $\mu$l of PBS in a glass vial on ice. With continuous mixing provided by magnetic agitation, a 10-fold excess of acetic anhydride relative to total reactive amine groups was added in 20 $\mu$l aliquots over the course of 15 min and the mixture allowed to stir for 1 hr. The progress of the reaction was monitored by removing aliquots of the reaction, spotting onto silica-coated thin layer chromatography plates followed by ninhydrin staining to detect the presence of free amino groups [232]. The acetylated peptide was analysed by MALDI and stored at 4°C without further purification.

[Diagram of acetylation reaction]

Figure 5.4: Acetic anhydride histone lysine acetylation

5.2.5 Solid-phase antibody binding assay to acetylated lysine

The lysyl side chains of BSA were acetylated by adding a 20-fold molar excess of acetic anhydride dropwise to a 10 mg/ml BSA solution in PBS on ice. The mixture was incubated for 2 hr and used without further purification. 40 nm diameter citrate-stabilised gold nanoparticles (500 $\mu$l, 1 nM) were coated with acetylated BSA (1 mg/ml) by electrostatic assembly [123] and purified by centrifugation (9,000 g, 10 min) and resuspension of the pellet in PBS. Dye-labeled anti-acetyl lysine antibody was spotted onto a nitrocellulose membrane and free binding sites blocked with BSA as described in Chapter 3. The membrane was incubated with the Ac-BSA/AuNP conjugate, diluted in a PBS solution containing 0.1 % (w/v) BSA to a final AuNP concentration of 100 pM for 3 hr at room temperature, followed by extensive washing in PBS and deionised water and silver enhancement, as described in Chapter 2.
5.3 Results

5.3.1 Substrate QD conjugation

In order to investigate whether the kinase assay principle could be translated directly to measure HAT activity, it was sought to conjugate the p300 substrate peptide directly to the QD surface. Incubation of QD with peptide at peptide:QD ratios 30-50:1 in 50 mM borate buffer (pH 9.6) failed to produce stable QD dispersions, as evident from visible particle flocculation following centrifugation (13,000 g, 3 min). As the peptide is lysine-rich and has strong cationic character, it was sought to neutralise the charge density on the peptide by conducting conjugation reactions (30:1 peptide:QD) at elevated pH using 50 mM NaCO₃ (pH 11.0). Under these conditions, the ability of the peptide to interact with the QD surface electrostatically should be limited thus favouring hexahistidine binding. These elevated pH conditions were conducive to stable conjugate formation, as indicated by a lack of pelleting following centrifugation or any obvious signs of macroscopic aggregation. These conjugates were further analysed by agarose gel electrophoresis as described (Figure 5.5). It was observed that at elevated peptide:QD ratios >35:1 were accompanied by marked decrease in photoluminescence intensity. This may reflect a precipitation of the QD material in the gel owing to limited compatibility with gel running buffer and/or the introduction of additional surface-trap states by the cationic peptide [233].

Further evidence was obtained to suggest that lysine residues were responsible for the difficulty in preparing the QD-conjugates and the observed quenching of photoluminescence by comparing the H4-peptide self-assembly characteristics with those of an acetylated derivative prepared by prior treatment of the peptide with acetic anhydride. This peptide did not cause macroscopic aggregation of QDs and the conjugates did not exhibit any significant photoluminescence quenching. In light of these observations, in summary, a ratio of 40:1 H4-peptide:QD was chosen to prepare the p300 substrate conjugates. It was essential to conduct the conjugation reactions at elevated pH to minimise particle flocculation induced by electrostatic charge screening by the peptide.
5.3.2 p300 HAT assay with QD-peptide conjugates

It was sought to determine whether the generic protein kinase assay protocol could be readily adapted to assay p300 activity. p300 was incubated with the H4-substrate conjugate in the presence or absence of 100 µM acetyl-CoA for 2 hr at 30°C before addition of a 10-fold molar excess of AF647-labeled anti-acetyl lysine antibody to QD. Numerous efforts failed to reveal sensitised AF647 emission and diminished QD donor emission indicative of FRET. This is somewhat surprising, as the overall dimensions of the QD-peptide should be comparable to the physiological nucleosome core particle from which the histone tails protrude [209]. The failure to observe evidence for p300 acetyltransferase-dependent FRET could be due to several possible reasons, for example i) the QD-dye distances are too great to observe FRET; ii) an inactive or insufficient amounts of enzyme limiting the acetylation; iii) non-functional antibody conjugates with compromised binding sites; iv) the antibody has insufficient accessibility to the QD-immobilised peptides; v) assay buffer interference; vi) lack of sufficient enzyme access to the QD-immobilised substrate. With these situations in mind, several additional control experiments were performed to identify the reasons for the lack of observable FRET.

5.3.3 MALDI analysis of enzyme reaction products

In order to verify that the p300 was catalytically active, reaction mixtures containing (20 µM) H4-peptide and 200 nM p300 in the presence or absence of acetyl-CoA were analysed by MALDI.
These experiments confirmed that the synthetic peptide could serve as a substrate for p300 when free in solution as suggested by the +42 and +48 increase in molecular mass, corresponding to the acetylation of one or two amine groups respectively (Figure 5.6). Furthermore, these observations are in good agreement with other reported MALDI data on p300 acetylation of H4-related peptides which also [234]. Thus it is possible to conclude that the enzyme is active, and the peptide can in principle serve as an enzyme substrate despite the presence of the appending hexahistidine tag. It was not possible to discern the precise sites of acetylation, however limited proteolysis with trypsin followed by peptide mass mapping could in theory help identify which lysine residues are targeted by the enzyme [235].
Antibody binding to acetylated BSA

A convenient and rapid test for functional anti-acetyl lysine binding was also developed to ensure that the antibody structure had not been compromised following dye modification. To this end a
heterogeneous assay was developed, with serial dilutions of AF647-labeled AcK antibody spotted onto a nitrocellulose membrane prior to blocking with 4 % (w/v) BSA. 40 nm citrate-stabilised gold nanoparticles were passivated with acetylated BSA and incubated with the antibody-coated membranes [123]. Successful binding was revealed following Ag⁺/hydroquinone silver enhancement in a [anti-AcK]-dependent manner, suggesting that the binding activity of the antibody had not been compromised (Figure 5.7).

Figure 5.7: Silver-enhancement binding assay for nitrocellulose-bound anti-AcK antibody and AuNPs coated with acetylated BSA. Duplicate two-fold dilutions of anti-acetyl lysine antibody (concentrations shown below) were spotted onto a nitrocellulose membrane prior to blocking of the membrane with BSA. Gold nanoparticles coated with acetylated BSA were incubated with the membrane to allow antibody capture. This was followed by extensive washing and subsequent nucleation of silver salt reduction on the surface-bound gold nanoparticles.

5.3.5 Anti-AcK binding to Ac-H4 peptide modified QDs

With the suggestion that the anti-AcK antibody had not lost its binding capacity, further controls were carried out to investigate whether the antibody was compatible with binding to chemically-acetylated peptides bound to QD surfaces. Acetic-anhydride modified peptides were allowed to self-assemble with QDs, as described, and the conjugates simultaneously separated from excess peptide and transferred into HAT assay buffer by centrifugal filtration. Addition of AF647-labeled anti-AcK resulted in a concomitant increase and decrease in AF647- and
QD-specific emission respectively in a concentration-dependent manner. This behaviour was not observed in the absence of peptide or where the non-acetylated H4 peptide was employed, suggesting that specific immunorecognition of acetyllysine was driving the FRET process. From the steady-state intensities, a maximum FRET efficiency of 53% was observed which suggested a possible upper limit to the signal response. In addition, inclusion of acetyl-CoA in the medium did not have any effect on the observed FRET efficiencies, confirming that its presence was not adversely affecting the integrity of the peptide-QD interaction.

Figure 5.8: Immunorecognition of acetylated peptide drives FRET. The acetic anhydride-treated peptide was assembled on the surface of the QD followed by addition of FRET acceptor dye-labeled antibody to achieve different QD:antibody stoichiometries. These binding assays were conducted in the presence of 100 µM acetyl-CoA to verify the coenzyme would not interfere with peptide assembly or antibody binding.
5.3.6 Enzyme steric accessibility and assay modification

Taken together, the above data suggest that the core components of the system (QD, peptide and cognate antibody) are individually functional but cannot respond appropriately to drive p300-dependent FRET, implying that the QD-peptide conjugate may be unable to serve as an efficacious substrate for the enzyme owing to steric hindrance attributable to the dimensions of the nanoparticle or excessive amounts of peptide on the surface leading to molecular crowding [135]. It is also possible that the broad distribution of positive surface potential of p300, as revealed in the high-resolution crystal structure [230], may result in electrostatic repulsion of the QD-peptide conjugate preventing the peptides from accessing the substrate binding pocket. Indeed, electrostatic interactions in enzyme-nanoparticle assemblies have been demonstrated to be able to play a key role in the augmentation of enzyme-substrate association and catalytic turnover [121]. Several possible approaches could be taken to remediate the apparent insufficiency in enzyme-substrate accessibility. For example, the peptide loading density could be reduced further, however this could limit the FRET response and the sensitivity of the system. It could also be possible to modify the substrate sequence with the incorporation of additional oligoethylene glycol (OEG) or peptide linker motifs to assist in presenting the peptide target sequence to the p300 active site. This approach, however, would further increase the interfluorophore separation and adversely affect the maximum possible FRET efficiency and is limited by the cost of heterobifunctional OEG reagents [236]. In order to circumvent fundamental redesign of the peptide substrate sequence, an alternative two-step assay methodology was investigated, whereby enzymatic peptide acetylation is allowed to occur in solution in the absence of QDs. Following the reaction, the acetylated peptide products are detected by assembling them upon the surface of the nanocrystals in the presence of the complementary anti-acetyl lysine antibody, resulting in immunorecognition and FRET (Figure 5.9). This approach is conducive to both efficient p300 acetylation and antibody binding as supported by the MALDI an FRET data respectively.
5.3.7 Multistep reaction procedure

Allowing the enzyme acetylation reaction to occur in a QD-free reaction medium prior to immunodetection resulted in FRET signals analogous to those observed in the case of the chemically-acetylated peptide. This effect was not observed in the case when acetyl-CoA was omitted from the reaction mixture, consistent with the hypothesis of p300-dependent acetylation of the peptides being responsible for FRET. BSA was included in the reaction medium to minimise non-specific binding and is commonly used as a buffer component in HAT assays [234]. Furthermore, despite the reasonably broad specificity of p300, BSA does not serve as a suitable acetylation substrate, as indicated by radioisotope labeling studies, and should not provide a source of assay interference [237]. It was anticipated that its presence may preclude peptide binding to the QD surface as it is believed to associate readily with the surface of MPA-capped nanocrystals by electrostatic interaction [173] [238]. Nonetheless, introduction of an aliquot of the HAT reaction mixture to a solution of QDs and antibody resulted in a rapid increase in the FRET ratio (670 nm/605 nm) which eventually reached a maximum value in c.a. 30 min (note the non-zero \( t = 0 \) time point owing to dead-time due to sample loading). Thus it can be tentatively concluded that the BSA does not inhibit peptide self-assembly upon the QD surface and can be incorporated into the assay medium without undue interference. Control experiments in the absence of acetyl-CoA were also carried out to verify the specificity of the process which did not exhibit the same increase in FRET ratio. These observations suggest that the modified
two-step assay format can be applied to detect p300 HAT activity and that enzyme access to the peptide substrate is severely impaired when bound to the surface of the QD. Whilst a two-step process avoids problems of steric hindrance, this format imposes some limitations, namely the increased number of reagent transfer steps renders the assay unsuitable for continuous real-time measurements. Nonetheless, this subtle modification allows the use of unbound peptide substrates which may more accurately mimic the behaviour of biological substrates.

![FRET signal evolution](image)

**Figure 5.10:** Time-resolved evolution of FRET signal following addition of acetylated peptide

In addition, aliquots of the p300 reaction mixture were transferred to QD-antibody detection solutions at time intervals to monitor the evolution of the FRET signal as a function of enzyme reaction time (Figure 5.11). No further increases dye-specific emission were observed at t > 60 min suggesting the reaction had gone to completion. Due to suggestions that the QD-H4 peptide could not serve as an effective surrogate substrate, it was presumed that upon binding of the peptide to the QD surface the transferase reaction would terminate due to steric hindrance, providing a convenient method to quench the reaction without need for additional inhibitors or chemical agents [207].
Figure 5.11: Photoluminescence emission spectra taken at 15 min intervals post-addition of 320 nM p300. The magnitude of FRET between QD and dye was monitored by taking the ratio of the emission peaks at 670 nm and 605 nm respectively.

5.3.8 Detection limit

The lower detection limit for p300 was determined to be 600 pM based on the concentration of enzyme required to produce a significant signal over a negative control (Figure 5.12). This was achieved using extended reaction times (6 hr) and is comparable to other radiometric methods, which are typically considered to be among the most sensitive detection technologies, such as liquid and flashplate scintillation (8 nM and 200 pM [239, 240]). Increasing this detection limit should be possible by increasing the dye:antibody ratio, thereby increasing the number of total energy acceptors per QD donor and boosting the maximum possible FRET signal. Unfortunately, dye:antibody ratios c.a. > 3.6 could not be prepared for the monoclonal antiacetyl-lysine antibody used in this study. In the future, the use of antibodies of different clonal origin or polyclonal antibodies which may be amenable to higher dye-loading capacities could boost this maximum possible FRET response.
5.3.9 Inhibitor response

It has been suggested that selective blockade of HAT activity, including p300, by small-molecule modulation may provide new therapeutic strategies for the treatment of a range of disease states [241, 207]. Anacardic acid, a cell-permeable analog of salicylic acid derived from cashew nut shell extracts, inhibits p300 HAT both in vitro and in vivo and has been proposed as an anti-tumour agent [242, 243]. Anacardic acid acts as a non-competitive p300 inhibitor, however, the precise structural basis of inhibition is not currently well understood [241]. To demonstrate that the p300 assay could be used to screen for inhibitors, the enzyme was pre-incubated with serial dilutions of anacardic acid predisolved in DMSO prior to addition of non-limiting acetyl-CoA and detection. Increasing concentrations of anacardic acid were predicted to correlate with a concomitant decrease and increase in emission intensities at 670 nm and 605 nm respectively due to reduced acetylation and QD-FRET immunocomplex formation. The measured emission intensities at 605 nm and 670 nm were used to yield the ratio 670 nm / 605 nm and plotted as a function of anacardic acid concentration (Figure 5.14). Consistent with predictions, this yielded

![Graph showing the ratio of 670 nm / 605 nm peak intensities for varying enzyme concentrations. Asterisks indicate significant signal above control (95% confidence level, n=3).]
the sigmoidal behaviour indicative of an inhibitor dose response curve and an apparent IC50 of 76 µM, corresponding reasonably well with the known IC50 of ∼8.5 µM as determined by radioisotope labeling [241]. An extensive literature review was unable to retrieve any further examples of IC50 measurements for further comparison and equipment limitations prevented further IC50 validation using alternative techniques (e.g. [3H]-AcCoA labeling). Nonetheless, this data suggests that the p300 QD-FRET assay can be used to measure the potency of selective inhibitor compounds.

Figure 5.13: Anacardic acid structure

Figure 5.14: Anacardic acid dose response curve. 670 nm /605 nm peak ratios showing effect of pre-incubation with varying concentrations of inhibitor. The data show a typical dose-response curve. Each data point represents the average of two independent measurements.
5.4 Conclusions and Future Work

It has been demonstrated that QD surface chemistry can be tailored to detect histone acetyl transferase activity, providing further evidence that a diverse range of post-translational modifications can be interrogated using QD-based approaches. It was sought to demonstrate that the assay principle reported in Chapter 4 for kinase activity could be readily applied to fundamentally unrelated families of enzymes without need for fundamental redesign of the system. In contrast to the kinase system, QD-peptide conjugates did not appear to serve as suitable surrogate substrates for the p300 HAT, possibly reflecting poorly defined steric requirements of the enzyme and/or inappropriate peptide orientation on the QD surface. This potential hurdle was overcome by conducting the p300 HAT assay with the substrate peptide free in solution in order to ensure uncompromised enzyme accessibility, followed by immobilisation on the QD surface and FRET-based immunodetection. In fact, this modified assay approach confers several advantages to the system in that no purification of nanoparticle conjugates is required and a more physiologically relevant substrate can be used as it is free to diffuse in solution. In addition, no elaborate chemical functionalisation or fluorophore labeling of the peptide substrate is required. This is particularly notable as fluorophore-labeled peptides may not necessarily provide accurate mimics of endogenous substrates for histone-modifying enzymes and provide misleading mechanistic information. For example, new inhibitors for the HDAC SIRT1 identified in a high-throughput fluorescence polarization assay were proposed to act as specific activators of the enzyme, however recent evidence suggests that the inhibitors interact directly with the TAMRA-labeled peptide substrate used in the assay [244]. The system developed here has numerous advantages compared to current state of the art and routine laboratory based HAT assays in that it does not require the use of radioisotope labels and can be conducted in a single homogeneous reaction, making the approach conducive to high throughput drug discovery. Similar to the protein kinase activity assay developed in the preceding chapter, the QD HAT assay is of sensitivity comparable to radiometric assays and can be operated using far-red emitting fluorophores, thus providing a convenient means to circumvent biological or test compound autofluorescence [50].

Given the combined evidence that QDs can be engineered to detect both kinase and HAT activity, it is not unreasonable to suggest that other post-translational modifications could be
detected using a similar approach, simply by changing the sequence of the peptide substrate and complementary antibody directed towards the specific covalent modification, (methylation, ubiquitinylation etc.) [245]. In addition, it would be pertinent to investigate whether the HAT system developed here could be applied to detect site-specific modifications and discriminate between acetylation sites on different lysine residues, as the physiological consequences of histone modification is often closely related to its specific chemical context. For example, acetylation of histone 4 (H4) K8 and K16 results in transcriptional activation, whereas acetylation of K5 and K12 induces histone deposition on DNA [214]. This could be achieved using the wide range of commercially available antibodies specific to individual histone modification sites which are used widely in cellular epigenetic analysis [210]. Furthermore, it is possible that the antagonistic deacetylase reaction (lysine-Ac → lysine-NH₂) catalysed by histone deacetylases (HDACs) could be probed using an analogous sensing procedure. HDACs are also considered to be highly promising drug targets for the treatment of a range of diseases and several inhibitor compounds are currently in clinical trials [246]. Such an HDAC assay could be realised using acetylated peptides based on histone tail sequences, prepared using standard solid-phase synthesis, and conjugating to the QD following treatment with a cognate HDAC. HDAC activity would presumably prevent anti-acetyl lysine binding due to loss of the corresponding antigenic site, leading to a decrease in the rate of QD-dye FRET.

As evolution has yielded a range of histone modification-specific binding domains, it may be possible to apply fluorescently-labeled recombinant versions of these domains to substitute for the use of antibodies in the assay; for example, acetylation-specific bromodomains [208]. In addition, using recombinant techniques, it may be possible to engineer specific residues (e.g. cys) in the binding domain to afford site-specific fluorophore labeling and thus better-defined fluorophore orientation. Highly selective DNA aptamers for acetylated histones have been developed which can discriminate between different acetyl lysine residues with high specificity and may provide another alternative to the use of antibodies [247].

It may be possible to realise some form of multiplexed assay for the detection two or more HAT enzymes in a homogeneous reaction, thus providing a means to screen enzyme-specific inhibitors. For example, two HATs with non-overlapping substrate specificity could be incubated with appropriate His₆-appended peptides in a homogeneous acetylation reaction to yield two
orthogonal acetylated peptides. This would be followed by self-assembly of the peptides on a QD FRET donor and detection of the acetylated products using two distinct antibodies with appropriately high specificity. FRET detection could be afforded by labeling the two different antibodies with appropriate acceptor dyes with similar absorption profiles and well-separated emission spectra in conjunction with spectral deconvolution.

Further work is now in progress in collaboration with the Imperial College Drug Discovery Centre to further validate the applicability of this assay as a lead discovery tool in automated medium- and high-throughput screening, potentially assisting in the identification of the next generation of cancer therapeutics based on targeting epigenetic modifications.
Chapter 6

Conclusions and Future Work

By integrating diverse elements of biochemistry, chemistry, pharmacology, materials science and photonics, this thesis has developed novel enzyme sensing strategies with potential utility as drug discovery tools, based on the unique plasmonic and photoluminescent properties of gold and semiconductor nanoparticles. Biofunctionalised nanoparticles were designed to undergo enzyme-dependent aggregation and participate in biospecific recognition processes to provide convenient colourimetric or fluorescence signals as a function of enzyme activity.

Chapter 2 aimed to demonstrate the protein kinase-dependent agglutination of antibody-modified gold nanoparticles to create a convenient homogeneous colourimetric assay based on interparticle plasmon coupling. It was determined that this approach had significant technical limitations due to complicated nanoparticle derivitization protocols, slow binding kinetics and susceptibility to non-specific binding. Nonetheless, it was demonstrated that these antibody-gold nanoparticle immunoconjugates may have utility in simple heterogeneous immunoassays based on silver enhancement amplification.

In order to address the limitations of the gold nanoparticle system, an alternative enzyme sensing strategy was developed based on energy transfer (FRET) between peptide modified quantum dots and dye-labeled antibody molecules. Rather than employing complicated, multi-step nanoparticle modification procedures, reliant upon expensive heterofunctional crosslinkers, water soluble quantum dots were conjugated to peptides based on known protein kinase substrates using a rapid self-assembly strategy as described in Chapter 3. This is the first demon-
stration that commercially available quantum dots can be rendered water soluble and conjugated to peptides under mild ambient benchtop conditions and should facilitate the development of future nanocrystal-biomolecule conjugates. In addition, these peptide-based nanoparticle conjugates could also be applied to simple immunocassays for target antigens in a heterogeneous immunoassay and in vitro imaging. Chapter 4 detailed how these peptide-QD conjugates can serve as substrates in a simple, generic, sensitive and rapid protein kinase activity assay based on immunorecognition-actuated FRET between QD-phosphopeptide donors and dye-labeled anti-phosphotyrosine antibodies. The assay has significant advantages over state of the art fluorometric approaches due to the spectral tunability of the system and myriad of QD-acceptor dye combinations from which to develop further kinase assays. This system is undergoing further optimisation and being used as an experimental basis for cellular QD-enzyme assays to distinguish between cells expressing a particular kinase and cells in which kinase expression has been selectively knocked down using RNAi [248].

Chapter 5 sought to extend from this assay principle to gain evidence that it was indeed generic and could be applied to distinctly unrelated enzyme systems. As a proof of concept, the assay was tailored to detect an unrelated enzyme family, the histone acetyltransferases (HATs), an emerging class of drug target candidates, as exemplified by the model enzyme p300. Initial experiments suggested that peptide-QD conjugates could not serve as efficacious p300 substrates in a manner analogous to the protein kinase system, possibly reflecting poorly-controlled peptide orientation on the nanocrystal surface or insufficient enzyme accessibility to the substrate sequence. This issue was circumvented by adapting the HAT assay in a multistep procedure whereby the enzyme-catalyzed acetylation reaction was initially conducted with the peptide free to diffuse in solution, followed by a detection step involving capture of the acetylated peptide on the QD surface and antibody-based FRET detection using dye-labeled antiacetyl lysine antibodies. In addition it is notable that fortuitously, this approach achieved one of the principle aims of the gold nanoparticle-based enzyme detection approach; namely the use of peptide substrates which remain unbound from the nanoparticle surface, thereby abrogating the necessity for complicated nanoparticle derivitisation whilst providing a more accurate mimic of natural substrates. This QD-based HAT detection approach had sensitivity comparable to state of the art radioisotope-based procedures, removing the need for costly and potentially hazardous
reagents such as scintillant-coated microplates and $[^3]$H-acetyl-CoA [240] [244]. It should be possible to readily apply this assay to high-throughput screening of small-molecule libraries for compounds which selectively inhibit p300 activity, thereby providing a new simple lead-discovery tool for potential therapeutics for diseases such as cancer, HIV and chronic inflammation [234, 226, 249]. Subtle modification of the system, for instance by changing the peptide substrate sequence to correspond to different histone-tail motifs (i.e. H2A, H2B, H3), should enable the detection of other related HAT enzymes with different substrate specificity. It is possible to envisage a highly multiplexed histone tail modification assay where four QDs with distinct emission profiles could each be conjugated to one of the four histone tail sequences, followed by subjecting to enzyme-catalyzed modification and detection with appropriate antibodies. This approach could provide a technique to probe the substrate specificity of histone modifying enzymes, however the ability to prepare stable QD-histone tail conjugates which can be readily accessed by enzymes is a fundamental prerequisite to the realisation of this potentially powerful yet complex hypothetical system.
Bibliography


Publications, patents and conference presentations


  - One of twelve articles selected from almost 1000 contributions to appear in a special “Best of” edition of the journal


- J.E. Ghadiali, S.W. Lowe, M.M. Stevens “Homogeneous Quantum Dot-based FRET Immunoassays for Histone Acetyltransferase Activity” (in preparation)

- J.E. Ghadiali, M.M. Stevens, “Particle-based assay” (PCT application no. PCT/EP2009/066121)

- “Quantum Dot / Gold nanoparticle Protein Kinase Assays” (2008) Materials Research Society, Fall meeting, (poster), Boston MA
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