

# Technology background

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**QTL Technology Introduction**

The polymer-QTL (Quencher-Tether-Ligand) approach is a single-step, instantaneous, homogeneous assay where the amplification step is intrinsic to the fluorescent polymer. The polymer-QTL approach provides a system for effective sensing of biological agents by observing fluorescence changes. The key scientific basis is the amplification of quenching of fluorescence that can be obtained with certain charged conjugated polymers and small molecule quenchers. In addition, the process is uniquely simple because there are no reagents.

A fluorescent polyelectrolyte-based superquenching assay has been shown to offer several advantages over conventional small molecule based fluorescence assays. For example, conjugated polyelectrolytes, dye-pendant polyelectrolytes, etc. can “harvest” light effectively both by absorption and by superquenching (1-5). The enhanced absorbing power of the polymers is indicated by the observation that even sub nanomolar solutions of some of these materials are visibly colored. The fluorescence of these polymers can be detected at even lower concentrations. Superquenching occurs

in the presence of small molecules capable of serving as electron transfer or energy transfer quenchers to the polymer or one of its repeat units.

### Polymer-Polymer Ensembles and Their Application to Biosensing

The fluorescent polyelectrolytes typified by compounds 1 and 2, show, in addition to their adsorption properties, a very strong tendency

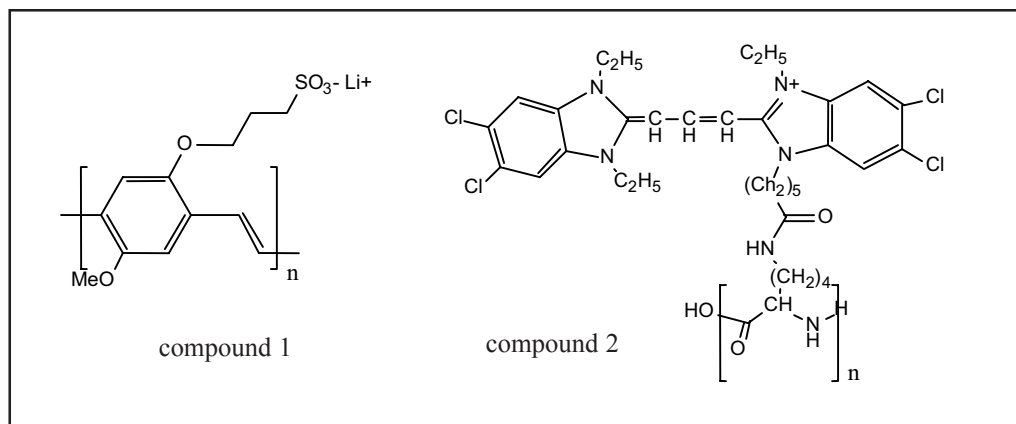
to associate with oppositely charged macromolecules, including other polyelectrolytes and each other. Compounds 1 and 2 and other polyelectrolytes in which the repeat unit or chromophore is varied provide a series of materials with absorption maximum wavelengths that span the range from the near ultraviolet to the visible-infrared, especially, for example, by varying the cyanine dye repeat unit in compound 2. The association of two oppositely charged fluorescent polyelectrolytes can lead to several interesting and potentially useful effects if we consider only as example compounds 1 and 2.

Thus, the association of nearly equimolar, in repeat units, amounts of compounds 1 and 2 results in an ensemble that is overall close to neutral, yet consists of discrete regions of negative and positive charges. Since compound 2 shows an emission at lower energies than compound 1, it is observed that energy transfer should occur. Thus, excitation into regions where the absorption should be primarily by compound 1 results in predominant emission by compound 2. Since compound 2 has a very sharp emission, the harvesting of energy within the ensemble provide possibilities to tune both the absorption and emission properties far beyond that which is available within a single polymer.

A most striking advantage obtained by using an ensemble such as the combination of compounds 1 and 2 is that both anionic and cationic small molecule quenchers can quench the overall near-neutral polymer mixture. As a result, it is observed that fluorescence from the ensemble is quenched

concentrations of either quencher such that the degree of superquenching shows only a slight attenuation compared to quenching of the individual polymers by the oppositely charged small molecule.

These results show that the polymer-polymer approach offers distinct advantages for biosensing by the polymer-QTL method. The polymer ensemble can be quenched by both positive and negatively charged QTL bioconjugates. Therefore, either in quench/unquench



formats or in a competitive assay, the polymer-polymer ensemble provides a means of obtaining higher selectivity and specificity. Furthermore, the degree of quenching by either cationic or anionic quenchers can be tuned directly by varying the stoichiometry of the polymer mixture. For example, when polymer 1 and polymer 2 are mixed in a ratio of 100:1, the superquenching by cationic QTLs is maintained and no quenching by anionic QTLs is observed. However, efficient energy transfer is still observed to polymer 2 even at this low ratio. By going to a 2:1 ratio of polymer 1: polymer 2, superquenching by both cationic and anionic QTLs is observed. Thus, charge tuning of the QTL assay is achieved by altering the stoichiometry of the anionic and cationic polymer. Both the net charge of the supramolecular cluster and the energy transfer characteristics of the combination may be tuned in this manner.

### Multiplexed Detection Using Mixtures Containing Supported Polymer

The interaction of anionic and cationic fluorescent polymers with each other can be eliminated by first anchoring one polymer to a bead or other supported format. For example, it has been demonstrated that anchoring polymer 2 to a clay suspension, prior to the addition of polymer 1 prevents the association of polymers 1 and 2. In this way, independent superquenching of each polymer can be achieved in a single solution upon addition of either cationic or anionic quenchers.

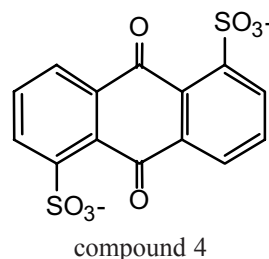
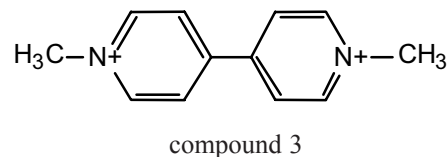
### Supported Formats for Monomers, Oligomers and Polyelectrolytes

Fluorescent polyelectrolytes, including conjugated and J-aggregate polymers, can be used for sensitive biodetection and bioassays in solution formats. The basis of this detection is the combination of the “superquenching” sensitivity of these molecules to quenchers of opposite or neutral charges with the synthesis of a quencher-recognition conjugate (QTL). One improvement of the polymer-QTL approach involves anchoring the fluorescent polymer onto a solid support via adsorption. Several advantages can result from this adsorption.

Fluorescent polyelectrolytes may be readily adsorbed from aqueous or mixed aqueous-organic solutions onto oppositely charged surfaces such as slides, plates, oppositely charged polymer beads (such as, quaternary amine-derivatized polystyrene or sulfonated polystyrene), and natural or synthetic inorganic supports such as clays or silica, charged membranes, or other porous materials. Once adsorbed onto these supports, the polymers retain their intense fluorescence as well as their sensitivity to specific quenchers. The fluorescent polymers incorporated into these formats may be used in advanced assays as described below.

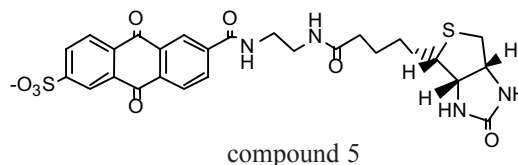
The incorporation of a fluorescent polymer onto a charged polymer bead can result in the reversal of the charge specificity in quenching of the polymer fluorescence as well as in improved performance in assays involving the polymer in either fluorescence quench or fluorescence unquench modes. In one example, the anionic conjugated polymer 1 is effectively quenched by low concentrations of the positively charged electron acceptor 3 in aqueous solution. However, its fluorescence is largely unaffected in solution by the addition of the negatively charged electron acceptor 4. When polymer 1 is treated with a

suspension of quaternary amine (cationic) derivatized polystyrene beads, the polymer is removed from solution and is irreversibly adsorbed onto the beads. In this



supported format, the highly fluorescent beads can be suspended in an aqueous solution and treated with the same quenchers. A reversal of the quenching sensitivity is observed; in the supported format, the anionic electron acceptor 4 quenches polymer 1, while the fluorescence of polymer 1 is no longer quenched by cationic electron acceptor 3.

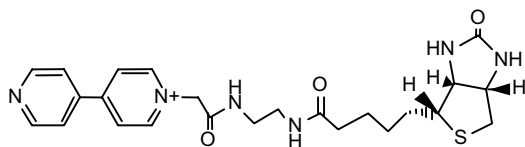
The charge reversal of fluorescence quenching can be adapted to biosensing by the polymer-QTL approach. Thus, QTL conjugate 5 which contains an anthraquinone quencher similar to anionic electron acceptor 4 and a



biotin ligand, is also observed to quench the fluorescence of polymer 1.

Upon addition of the protein avidin (a specific receptor for biotin), the quenching produced by conjugate 5 is reversed and virtually complete recovery of the fluorescence of polymer 1 is observed.

This contrasts with aqueous solutions where a viologen-based conjugate 6 has been shown to elicit a similar quench-recovery response with polymer 1. For both



compound 6

polymers 1 and 2, when dissolved in aqueous or partially aqueous solutions, nonspecific effects are frequently observed upon the polymer fluorescence by addition of macromolecules, particularly proteins leading to either partial quenching or enhancement. These interactions may occur with analyte proteins or with proteins not anticipated to interact with the specific QTL conjugate employed in the sensing and may interfere with specific effects due to the interaction of an “analyte” protein with the polymer QTL complex. These nonspecific effects may be eliminated or attenuated by employing polymers in supported formats.

A second example involves the use of a QTL conjugate, which quenches the fluorescence of polymer 1 by energy transfer. While the anionic energy transfer QTL does not quench the fluorescence of anionic polymer 1 in pure aqueous solutions, adsorption of polymer 1 on beads results in its quenching upon the addition of this conjugate and fluorescence recovery upon addition of avidin, the protein binding the ligand portion of the energy transfer QTL.

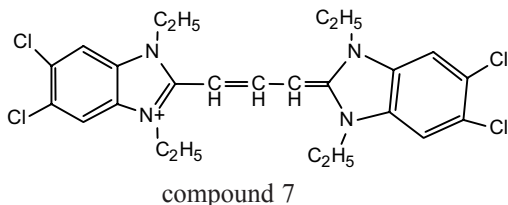
Adsorbing a fluorescent polymer on a charged support may not always lead to charge reversal in the quenching of the polymer. The charge reversal, or lack thereof, can be tuned by the degree of “loading” of the polymer onto sites on the support. In a different example, it is demonstrated that enhanced quenching can be obtained for a supported polymer as a consequence of adsorption. Thus, when cationic polymer 2 is adsorbed onto anionic Laponite clay particles, the polymer fluorescence is still subject to quenching when small amounts of anionic acceptor 4 are added to the aqueous suspension. Under these loading conditions, polymer 2 is not quenched by cationic acceptors such as compound 3.

Quantitative analysis of the extent of quenching by compound 4 under these conditions indicates that the clay-supported polymer 2 is quenched more effectively (in this

example by more than 30%) than when it is in a pure aqueous solution. This example illustrates two concepts that lead to improved biosensing with the polymer-QTL approach using supported polymers. The first concept is that the supported polymer can be used to “sense” oppositely charged quenchers when supported on the clay particles and yet exhibit improved stability with respect to degradation and precipitation (observed for aqueous solutions). When the same polymer is supported on the clay at lower loading levels, its fluorescence is quenched by cationic compound 3, thus demonstrating a charge reversal similar to that cited above with polymer 1. The second concept from these experiments with clay-supported polymer 2 and its quenching by compound 4 is that increased quenching sensitivity can be obtained due to polymer-polymer association effects on the clay particles. This increased quenching sensitivity may result from an increase in the J-aggregate domain (or conjugation length for conjugated polymers). The combination of enhanced quenching sensitivity and the ability to tune the quenching sensitivity in supported formats as described above greatly extends the potential of the polymer-QTL approach both in regards to sensitivity and versatility. Additionally, the anchoring of fluorescent polyelectrolytes on beads, surfaces, or membranes can expand the utility of the polymer-QTL approach. Thus, the strong adsorption of the polymers onto beads or membranes can provide detection of analytes in a “flow-through” mode using either liquid or vapor streams. Additionally, the tethering of the polymer onto plates in a multi-well array format by adsorption demonstrates the use of these formats in high throughput screening and rapid sampling applications. Furthermore, the elimination of nonspecific effects upon anchoring to a bead surface greatly enhances the practical usage of QTL-based assays.

### Virtual Polymers based on Covalent Attachment of Supramolecular Building Blocks.

Enhanced superquenching provides a new means of obtaining superquenching from much smaller oligomers and even monomers in an adsorbed format. Thus, it is possible to synthesize polymer 2 in a range of repeat unit sizes varying from  $n = 3$  to  $n = 1000$ . It would be anticipated that, to a first approximation, in solution, the higher molecular weight polymers should exhibit higher quenching efficiencies due to an “amplification factor” that should be directly proportional to the number of repeat units (6). However, as the number of repeat units increases, the solubility of the polymer decreases and the complexity of the polymer allows new channels for nonradiative decay to attenuate the effectiveness of quenchers. Therefore, in the case of polymer 2, the potential for attaining maximum sensitivity by using very high molecular weight polymers cannot be recognized.



The use of smaller oligomers (or even monomers, such as 7) in an adsorbed format permits the construction of effective higher order polymers by the formation of extended aggregates that bridge across adjacent polymer (or monomer or oligomer) molecules. This provides for enhanced levels of superquenching and thus new sensors of greatly enhanced sensitivity.

Assembly of cyanine dye monomer 7 or oligomers having the same structure as 2 but fewer repeat units on silica or clay nanoparticles results in the formation of “J” aggregates that exhibit high superquenching sensitivity (i.e., surface activated superquenching) to ionic electron transfer or energy transfer quenchers. This can be attributed to a combination of high charge density (and resulting Coulombic interactions) and excitonic interactions within the self-assembled units. These assemblies also can be used as biosensors in the QTL fluorescence quench-unquench mode. These virtual polymers can be easily assembled from a variety of monomer or small building blocks, often bypassing difficult steps of polymer synthesis, purification, and characterization. Although studies to date have shown self-assembled virtual polymers to be relatively stable with little sensitivity in their fluorescence to added macromolecules, it is clear that the small adsorbed units

may be subject to desorption or rearrangement under certain conditions, most notably high ionic strength. An approach that combines the simplicity of using small building blocks assembled on a surface with a more robust analysis platform involves the covalent tethering of monomers on the surface of a neutral or charged nanoparticle, bead, or other rigid support.

In one example, a relatively simple synthetic scheme similar to that developed for the cyanine poly-L-lysine 2 was employed in the construction of cyanine dye 7 covalently attached to the surface of 0.2  $\mu\text{m}$  diameter silica microspheres. The cyanine dye thus linked to the microsphere surface was found to exist both as small clusters of the monomer and as highly ordered aggregates. Efficient exciton migration/energy transfer between the dye clusters and aggregates was observed when the material was suspended in water containing 2% dimethylsulfoxide. The suspension also showed a significant reduction in emission intensity in the presence of anionic quenchers, indicating that superquenching of the covalently-linked dye assemblies occurs.

The modes of interaction between cyanine dye monomers on the microsphere may be controlled by varying the density and structure of functional groups present on the surface. Thus, the efficiency of biosensing can be optimized. Similar schemes may be used to append other cyanine dyes and other building blocks such as conjugated polymer oligomers onto a bead, particle, or other solid surfaces.

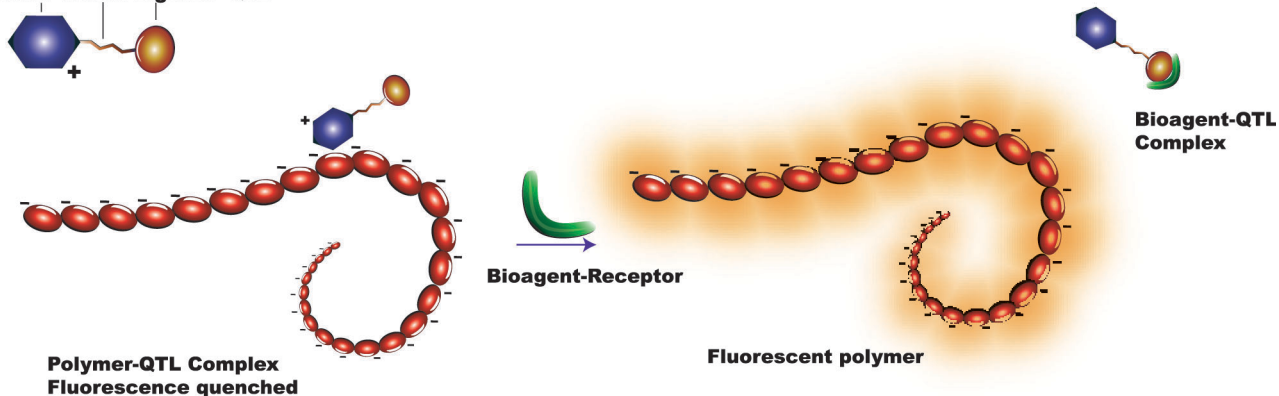
## Assay formats

### Fluorescence turn-on assay

A fluorescence based assay is realized when the QTL conjugate is used to quench the polymer either in solution or in supported formats at solution-solid or solution-particle interfaces (1,7,8). For example, fluorescent polyelectrolytes, including conjugated and J-aggregate polymers, can be used for sensitive biodetection and bioassays in solution formats. The basis of this detection is the combination of the “superquenching” sensitivity of these molecules to quenchers of opposite or neutral charges

with the synthesis of a quencher-recognition conjugate (e.g., a QTL molecule). In the original formulation, the QTL conjugate quenches the polymer ensemble by nonspecific binding (figure 1). Addition of a target bioagent capable of binding with the L component of the QTL conjugate results in a removal of the QTL conjugate from the polymer and a turning on of the polymer fluorescence.

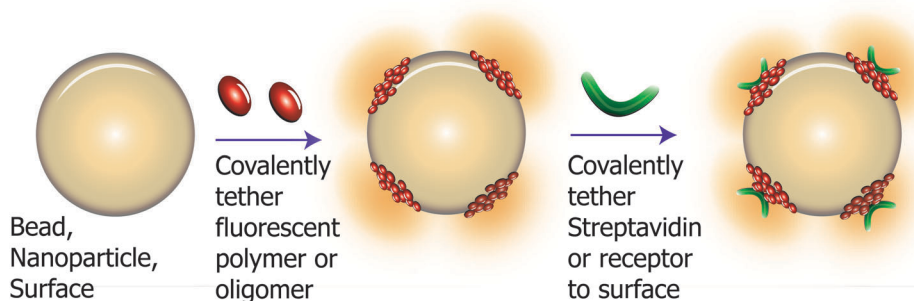
#### Quencher-Tether-Ligand: "QTL"



### Fluorescence turn off assay

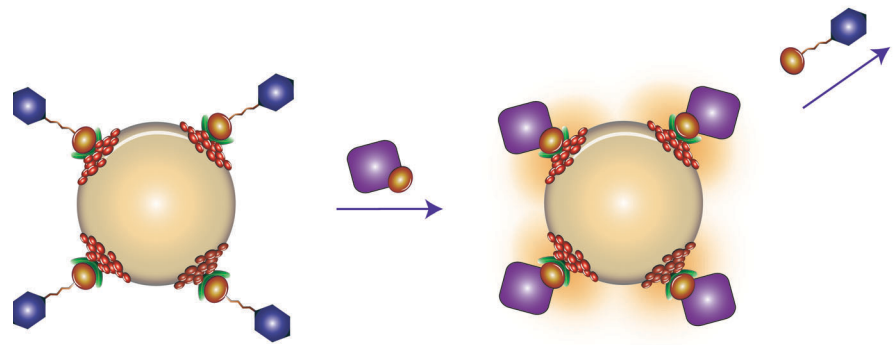
A fluorescence turn off (or modulation) assay has also been developed based on polymer superquenching (5). In this case, the target molecule is a bioagent L, or L', corresponding to the L component of the QTL conjugate, and the receptor is a biomolecule that strongly associates with L, L' or the QTL conjugate. One example is a direct competition assay in which L (or L') in unknown amount is allowed to compete with the QTL conjugate for the binding sites of a measured amount of the receptor. The polymer fluorescence is quenched by non-bound QTL to an extent depending on the amount of L (or L') present. In another example, the QTL conjugate is preassociated with the receptor; when all of the QTL conjugates are associated with quenching is observed. Addition of L (or L') to the sample results in the release of the QTL conjugate with concomitant quenching of the polymer fluorescence.

This assay formats depend on nonspecific quenching of the polymer fluorescence by association of the QTL conjugate with the polymer. A complication with these assays is the competing nonspecific interactions of other components of the assay sample with either the polymer, the QTL conjugate, or both, which result in a modulation of the quenching. To establish more specific and more robust assays we have adopted formats wherein a bioreceptor and a fluorescent polyelectrolyte or “polymer ensemble” are co-located on a bead, microsphere, nanoparticle or other surface. Several of these format-platforms are shown in the following sections. Figure 2 shows one way in which such a platform may be constructed.



### Displacement Competition assay

In the Displacement Competition Assay, the anchored fluorescent polymer-receptor is pretreated with the QTL conjugate, resulting in the binding of the QTL conjugate to the receptor and concurrent superquenching of the fluorescent polymer. As shown in Figure 3, the actual analysis involves the addition of an analyte to the ensemble. The fluorescence of the polymer increases quantitatively (turn on) with the level of the target agent in the analyte sample. Targets include proteins, viruses, bacteria, spores, cells, microorganisms, antibodies, antibody fragments, nucleic acids, and toxins. In this example, the assay may be homogeneous and the actual time for the assay may be controlled by the "off rate" of the QTL from the receptor.

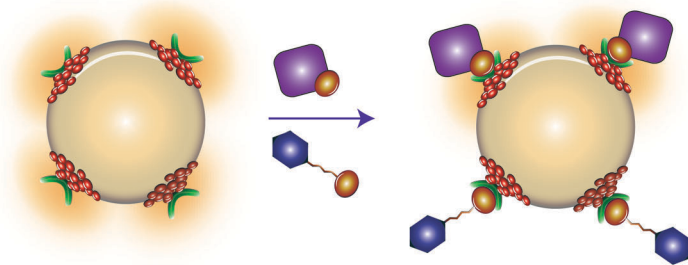


Analyte displaces QTL for "turn-on" competition assay

fig. 3

### Direct Competition Assay

As shown in Figure 5, in the Direct Competition Assay, the anchored fluorescent polymer-receptor is treated with a mixture containing an analyte (an unknown amount of the target agent) and a known amount of QTL conjugate. The polymer fluorescence is quenched to an extent determined by the QTL:target agent concentration ratio. The stronger the fluorescence, the higher the concentration of the target agent. An advantage of this approach used is that the assay may be both homogeneous and near instantaneous. Since both the target agent and the QTL conjugate compete directly for "open" receptor sites, the response can be very rapid. In another formulation, the anchored fluorescent polymer-receptor is incubated with an analyte sample before the fluorescence intensity of the sample is measured. The sample is then treated (following rinse steps as necessary) with an excess of a QTL conjugate. The initial reading of fluorescence following treatment with the QTL conjugate shows quenching due to binding of the QTL conjugate to unoccupied receptor sites. The stronger the initial fluorescence quenching, the smaller the level of target agent. Monitoring the polymer fluorescence as a function of time provides additional confirmation of the binding of the target agent and its replacement by the QTL conjugate at the receptor.



Direct competition assay

fig. 4

### A “turn on” Competitive Assay based on Polymer-Biomolecule Combinations.

Polymers that contain reactive end groups may be covalently linked to a variety of materials, including small molecules, other polymers, and biomacromolecules. The resulting “hybrid molecule” may have similar solubility and will generally have the same ability as the individual polyelectrolyte component to adsorb to a surface. These surfaces include slides or plates, oppositely charged polymer beads (such as, but not limited to, quaternary amine-derivatized polystyrene or sulfonated polystyrene), natural or synthetic inorganic supports such as clays or silica, charged membranes, semiconductor nanocrystals, and other porous

materials. Thus, either independently or as a component of a mixture, the use of a hybrid molecule can afford the preparation of a supported assembly containing a highly fluorescent species subject to superquenching. The hybrid molecule may also be employed in a solution-phase assay.

Sandwich assays may also be used in these assays as diagrammed in Figure 5.

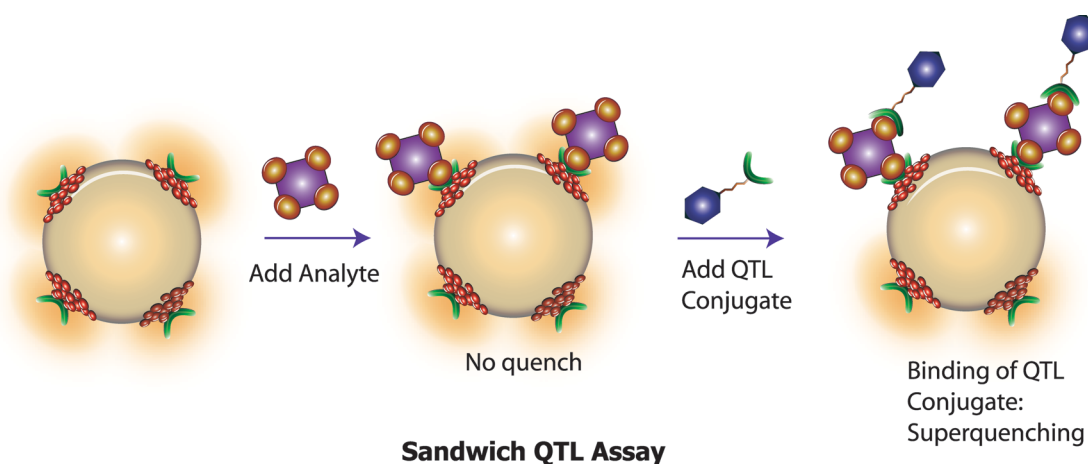


fig. 5

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