Miniaturization of Microarray based Screening of HSP inhibitors using nanoparticles and synthesising nanocarrier for inhibitors

A DISSERTATION

Submitted in partial fulfillment of the requirements
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in
NANOTECHNOLOGY

By
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CANDIDATE'S DECLARATION

I hereby declare that the work presented in dissertation entitled **"Miniaturization of Microarray based Screening of HSP inhibitors using nanoparticles and synthesising nanocarriers for inhibitors"** submitted in partial fulfillment of the requirements for the award of degree of **Master of Technology** in **Nanotechnology**, **Indian Institute of Technology Roorkee**, is an authentic record of my work carried out under the supervision of **PD Dr. Carsten Zeilinger,** Institute of Biophysics, BMWZ, Leibniz University Hannover, Germany and **Dr. P. Gopinath**, Associate Professor, Department of Biotechnology, IIT Roorkee. The matter embodied in this has not been submitted by me for the award of any other degree.

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CERTIFICATE

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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Abstract

Folding of protein is carried out by molecular chaperones like Heat Shock Proteins (HSP), out of which HSP70 and 90 are most vital members. Expression of HSP70 and 90 surges by a variety of cellular stresses. Human cancer cells display comparable stress symptoms. On a molecular level, this leads to a surplus of HSP in cancer cells, especially HSP90. HSP has ATP dependent protein folding action. Small molecules can interact with HSP by binding to nucleotide binding site, thus hampering folding of proteins. This leads to the breakdown of many activities in oncogenic cells. Few molecules have been recognized, that attaches to the ATP binding site. Microarray systems can be used to test in-house library of new inhibitors of HSP. Q-Dots have interesting electrical and optical properties. Exploitation of its surface plasma resonance effect for optical sensing plays a momentous role in the biomolecular diagnostic assay. Cancer nanotherapeutics are rapidly developing and being applied to solve several restrictions of traditional drug delivery systems such as nonspecific binding, low bioavailability, reduced solubility in aqueous media, truncated therapeutic indices and generalized biodistribution. The nanoparticle is in optimum size range and has unique surface characteristics which lead to prolonged circulation time in blood. They serve as carriers to drug molecules with high specificity for tumours, because of their enhanced permeability, amplified retention effect, and microenvironment of tumour cells. This thesis work aims at miniaturizing the microarray assay using Streptavidin conjugated Quantum-Dots. Q-Dots are spotted on Nitrocellulose surface and quenching of their signal by HSP attached to biotinylated-ATP so that it can be further used for screening inhibitors that bind to nucleotide binding site of HSP. It also aims at synthesizing HSP inhibitor loaded niosomes that can be used for targeted delivery to cancer cells.

Keywords

Streptavidin coated Quantum Dots, Biotin-ATP, Heat shock protein, HSP Inhibitor and Niosomes.

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1. Introduction

Heat shock proteins (HSPs) are a big class of proteins which exist in prokaryotic and eukaryotic organisms. They have been conserved throughout the evolution. Heat shock proteins have a significant part in protein homeostasis. They are instituting in all major cellular compartments. The most important activity in a cell under the stressed condition is the production of Heat shock or Stress proteins. This is an adaptive response that helps in cellular homeostasis under stress conditions. If there is a sudden increase in temperature or other metabolic or environmental stresses cells show induced expression of HSPs.

The mechanism of their expression and activation is as follows-

- Protein Denaturation occurs during stress condition.
- Presence of denatured protein (DP) leads to dissociation of the HSP-HSF (Heat Shock Factor) complex.
- Free HSP binds to denatured proteins while HSF gets activated by undergoing phosphorylation and trimerization.
- Activated HSF binds to the Heat Shock Element
- It is present in the promoter region of the HSP gene
- It induces its transcription.
- The newly formed HSP bind to more denatured proteins.

HSPs of Mammals have been classified into six groups based on their molecular size: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs including HSP27. Larger HSPs have ATP-dependent function while smaller one works in ATP independent way. The HSP90 is important in the development of the steroid receptor complex. The HSP70 family is essential for protein synthesis, translocation, and folding. HSP60 family is significant in protein stability.[1]

The cryoprotective mechanism of HSPs is of Molecular chaperone, the Apoptosis inhibitor, and protein stabilizer. As molecular chaperon HSPs catalyse the appropriate folding of misfolded proteins and evade their aggregation. As anti-apoptotic protein HSPs interfere with cell death at different stages. At post mitochondrial level HSP 27 binds to cytochrome c while HSP90 or HSP70 binds to the apoptotic protease activating factor 1(Apaf-1), thus inhibiting caspases activation and apoptosis. HSP 70 association with apoptosis-inducing factor (AIF) blocks the caspase-independent cell death. HSP gives stability or proteasomal degradation of proteins under stress (protein triage process). Thus, HSP contributes to cell survival.[2]

These cryoprotective functions of HSPs make cancer cells dependent on them as cancer cells must extensively rewire their metabolic and signal transduction pathways. Expression or activity of HSP90, HSP70 and HSP27 is abnormally high in cancer cells and other death stimuli increase it further. HSP70-I confer tumorgenicity in cancer cells, make them resistant to anti-cancer drugs like Cisplatin or Imatinib and it renders cancer cells resistant to the immune response from cytotoxic cells like monocytes. Antiapoptotic activities of HSP90 helps cancer cells in survival.[3]

The curiosity for HSP inhibitors has lately found an extra motivation, because of heat shock proteins can also serve as a target in the treatment of various pathogenic diseases, such as malaria and Leishmania, as well as neurological disorder.

Natural HSP inhibitors bind to the ATP binding pocket of the protein and prevent the ATPase activity. Thus, chaperone activity is inhibited. For example- Radicicol isolated from fungus *Monocillium* and Benzoquinone, Ansamycinan and Geldanamycin. Geldanamycin has high hepatotoxicity thus, it has been suspended from the phase I trial. Its analogue like 17-AAG are the first-in-class inhibitor and have entered phase II trials. AIF derived peptides which are rationally designed decay targets of HSP70 sensitize cancer cells to apoptosis induction. VER-155008 is adenosine derived compound that fits in the ATPase pocket of HSP70, inhibits its chaperone activity and induces caspase-dependent apoptosis. HSP70 antisense oligonucleotides enhance apoptosis in cancer cells and sensitize them to chemotherapy and radiotherapy.

Protein microarrays are growing as beneficial tools for biopharmaceutical research. These micro spot arrays have a boundless potential to fast-track not only elementary biological discovery but also drug development. Quantum dots in microarrays, offer very exclusive features that allow detection at pg/ mL concentration. Quantum dots offer notable photostability and brightness. They do not display photobleaching common to organic fluorophores. The high emission amplitude for QDs results in a better signal to noise ratio of the final image. Microarrays allow highly parallel detection and quantification in a rapid, low-cost and low sample volume format.[4]

In this project quantum dots are used for designing microarray assay. Quantum dots conjugated with Streptavidin are spotted on nitrocellulose membrane. To which Biotin-ATP is attached. Heat shock protein solutions with and without inhibitor are added to the microarray. In absence of inhibitor Heat shock protein attaches to the ATP and mask the fluorescence of quantum dots. In presence of inhibitor Heat shock protein does not attach to ATP hence there is less masking. In this way we can test compounds as inhibitor for HSP.

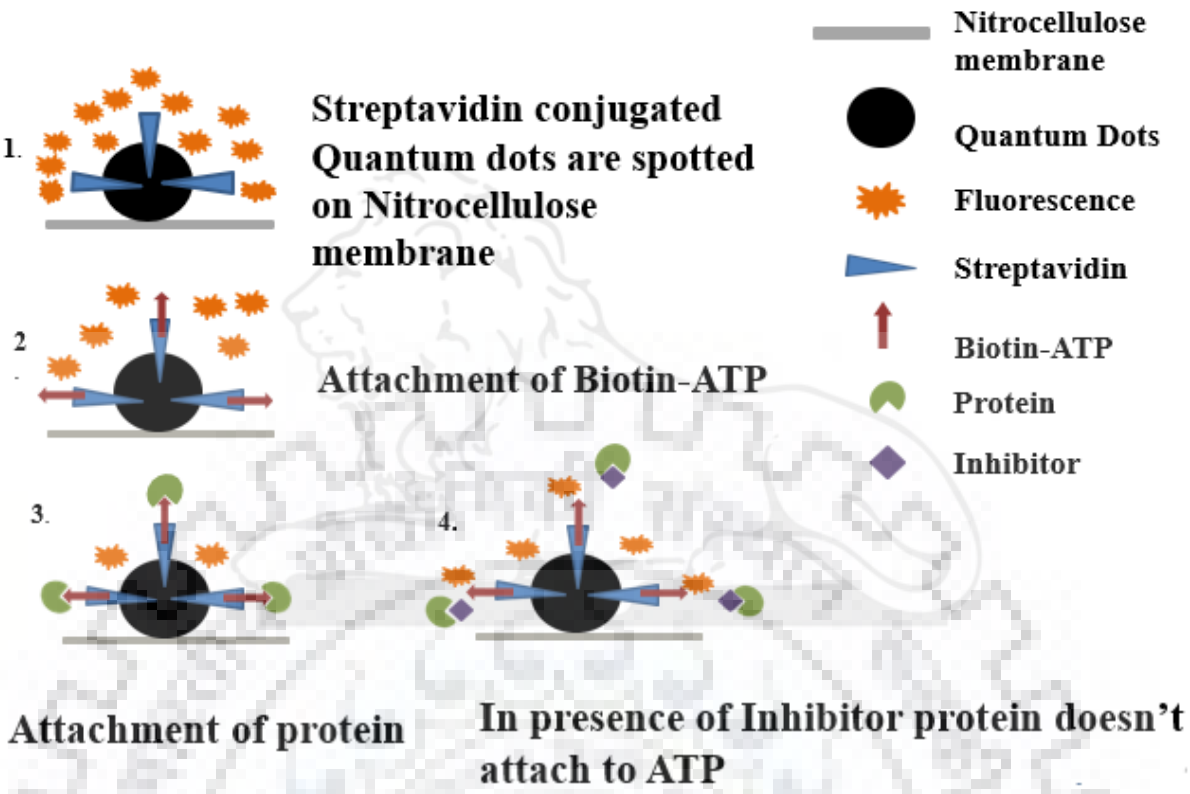


Figure 1. Graphical abstract of miniaturization of assay

HSP inhibitors like 17-AAG lack oral bioavailability and have poor solubility. Low concentrations of HSP90 inhibitors may enhance angiogenesis, whereas high concentrations of HSP90 inhibitors are antiangiogenic, retarding the formation of new vasculature in cancerous tissues. Therefore, targeted delivery of the HSP inhibitors like Geldanamycin and its derivatives using niosomes to tumours and limited distribution to other tissues will decrease nonspecific toxicities and improve efficacy. This may increase the residence time of encapsulated compounds in blood, and favourably increasing accumulation in solid tumours by the enhanced permeability and retention effect (EPR).[5]

Niosome is molecular cluster formed by self-aggregation of non-ionic surfactants in an aqueous phase. Niosomes gives prolonged, targeted and effective drug delivery system with the ability to pack both hydrophilic and lipophilic drugs. The surface formation and modification can be easily done because of the functional groups on their hydrophilic heads. Due to their non-ionic nature, they have low toxicity.

In this project niosomes are synthesised by injecting and aqueous and lipid solution into microfluidic channel. Size of niosomes are varied by changing flow rate of both solutions.

1.1 Objectives

- Miniaturization of the Microarray screening process using Streptavidin conjugated Quantum dots.
- To synthesize and characterize Niosomes that will serve as carriers of the inhibitors.

2. Literature review

In humans for most of the biological functions around 20,000 to 25,000 proteins are accountable. Proteins become functional after folding into three dimensional structures. Most of the freshly translated proteins fold simultaneously on their own. Small amounts of large complex proteins are susceptible to misfolding. This problem is increased in a crowded cellular environment. This happens only when complex proteins show their hydrophobic amino acids to the solvent, leading to unwanted interactions and aggregation. To prevent these cells has molecular chaperone. It is a protein that helps in folding or assembly of other proteins without being a part of final structure. They help in maintaining de novo synthesis and maintaining native state of proteins. Major function of chaperone is to avert aggregation of protein under cellular stress.[6–8]

Molecular chaperone helps wild p53 to reach its tumour suppressor potential. Mutated p53 which have hotspot for missense for mutations are stabilised by same chaperones into pseudo aggregates which are folding intermediate. These escape proteolysis quite easily and are harmful to cells. In tumour cells chaperones are reshaped and they rewire signalling pathways to benefit tumour progress.[9–11]

Heat shock proteins help cells in dealing with intrinsic and extrinsic stress. The general concept about stress is that it is a condition that disturbs normal functioning of a system or reduces the fitness level of organisms. Environmental factors are extrinsic stress factors and genetic problems and mutations are intrinsic. Heat shock protein genes were discovered on the chromosomal puffs of drosophila when they were exposed to high temperature. These genes are highly conserved, and their coding regions have very less variations among species. This and their universal presence show importance of HSP in cellular protection.[12,13]

HSPs hinder the action of pro-apoptotic Bcl-2 proteins to avoid permeabilization of the outer mitochondrial membrane and release of apoptogenic factors. One more way by which HSPs can avert caspase activation and initiation of apoptosis is by degrading formation of apoptosomes. Numerous signalling cascades are involved in the regulation of important elements in the apoptotic cascade are exposed to changes by HSPs, like JNK, NF- kB and AKT. [14,1516]

HSPs help bacteria to proliferate in more extreme environmental conditions. Increased temperature causes damaging effect to cellular proteins. HSPs with their cryoprotective functions protects the cell. This helps them to survive change from environment to host body.

Also, similarity with human HSP helps with interaction with immune cells and lead to autoimmune response which leads to cancer or leprosy.[17,18]

The HSPs are classified based on their molecular weight. The smaller family have level 11 members that includes HSP27. Their weight is around 40kDa and they are ATP-independent. This family is encoded by HSPB gene. The largest family encoded by HSPJ gene is HSP40 family. There are 49 members. HSP 10 and 60 are included in chaperonin family. Gene for HSP 70 is HSPA and it has 13 members. HSPC is gene for HSP 90 with 5 members. Larger ones are HSP110 and GRP170.[19]

HSP70 family is highly conserved in most of the organisms. It has 44kDa N terminal domain which has ATPase function, 18kDa middle domain for substrate binding and C terminal domain of 10kDa. It helps in de novo folding of proteins, prevents protein aggregation during folding of complex structure, channelling proteins to organelle and immunomodulatory functions. In the last function HSP cross presents peptides via MHC antigens or act as chaperokines.[20]

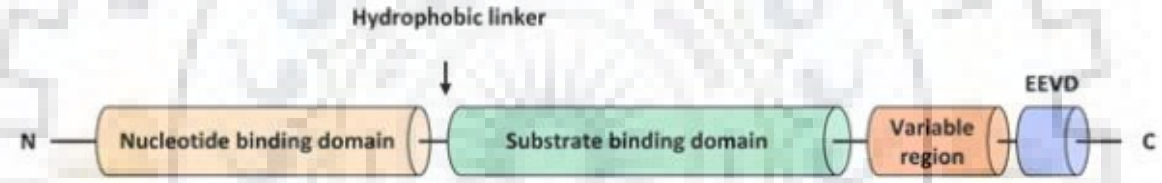


Figure 2. Schematic representation of HSP70[15]

Main location for HSP 90 is cytoplasm. N terminal domain is of 25kDa, middle is 40kDa and C terminal domain is 12kDa. N domain is homologous to ATPase/Kinase family. It helps in stabilization and regulation of cellular functions and activation of its client proteins like Kinase, Steroid hormone receptors, transcription factors etc. HtpG is its homologous in bacterial cell.[21,22]

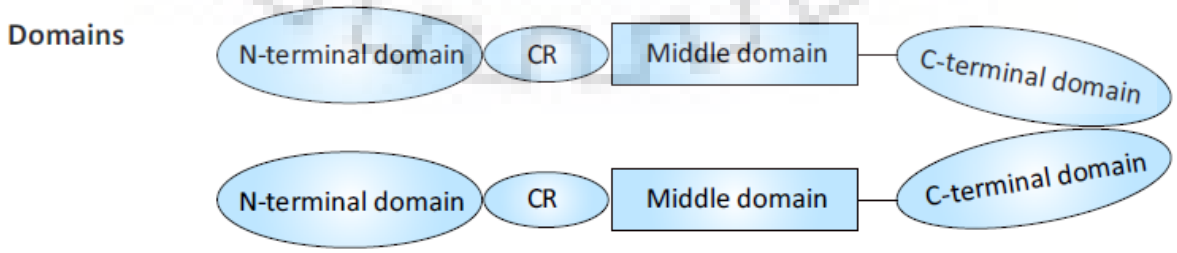


Figure 3. Schematic representation of HSP90[16]

Major role of HSPs in cancer cells is to stabilize the overexpressed mutated gene products. HSPs help in proliferation of cancer cells, development of resistance to treatment and ones released from cancer cells improves malignancy.[23]

Role of HSP90 in cancer development is regulating tumour growth, mobility, adhesion, angiogenesis, apoptosis and metastasis. Inhibition of HSP90 leads to decrease in angiogenesis and mobility in bladder cancer. HSP70 increases cancer potential of a cell by helping to immunological escape. It also helps with its increases tumour associated protein expression and evasion of apoptosis.[3,24]

Targeting chaperone activity specially of HSP90 has become a trend in recent years for developing drugs for cancer and other diseases that depends on molecular chaperone. ATPase activity is one of the main targets. Natural products like Geldanamycin, Ansamycin and their derivatives are in clinical trials. These are quinone based drugs and have high toxicity. There is a need to search new natural inhibitors for HSP.[25,26]

Protein microarray is developed by spotting purified protein on nitrocellulose followed by blocking rest of the surface. Cy3 or Cy5 labelled ATP is attached to the protein. In the presence of inhibitor there is competitive binding between inhibitor and labelled ATP. This way we can have competitive assay for natural products. [25,26]

Multiplexing system can be developed by spotting different HSP protein even from different organisms with ATP binding site as target. Dye labelled ATP is displaced by inhibitors. This allows testing of large number of potential drugs against various types of Heat shock proteins as well as proteins which are dependent on ATPase activity.[27–29]

Even cell lysates can be spotted on the microarray for targeting HSP present in them. This way elevated level of HSPs can be shown in diseased cells. Microarray assay can be used for detecting levels of HSPs as biomarkers for stress and diseases.[27,30]

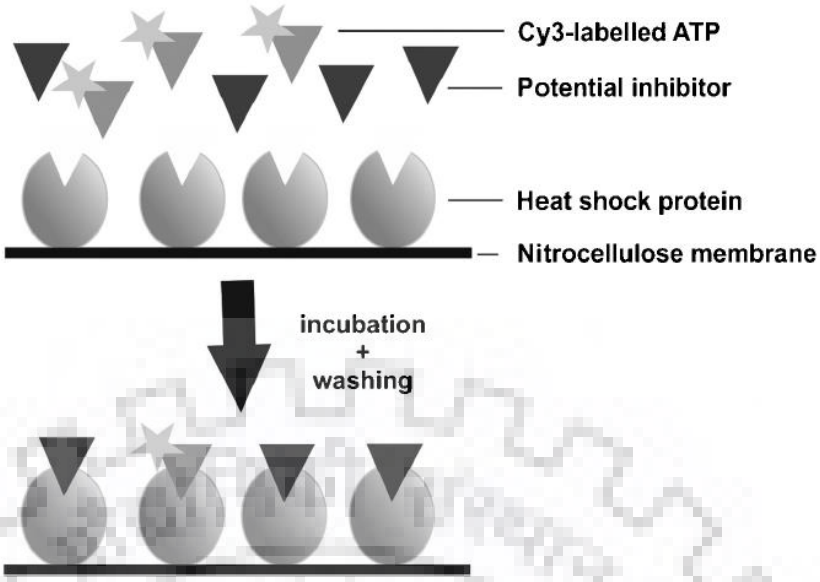


Figure 4. Schematic for protein microarray[23]

All the dye labelled ATPs aren't suitable for every type of Heat shock protein. Position of dye is important for different proteins. In case of HSP70 in the dye is attached at Phosphate tail then binding of ATP will not occur as the tail needs to be buried in the small binding pocket. If dye is attached at N8 position of base, then for displacement of attached ATP becomes difficult. For HSP90 dye attached to the nucleotide ring causes problem in similar way as for HSP70. [4,31,32]

Spotted protein microarrays can be stably stored for one month at 4°C. Although there is certain decrease in IC_{50} value for same inhibitor.[25]

Quantum dots are tiny semiconductor crystals. They have broad excitation range and size tuneable narrow emission wavelength. This is due to quantum confinement effect. It has a core shell structure. Core is made up of CdSe that absorbs and emit radiation while shell is made up of Zns. Shell layer is transparent i.e. it has high band gap, it has structure like core to confine excitement till core. Shell increases quantum yield. Conjugated Quantum dots like Streptavidin conjugated are used in detection system. 5-10 molecules of Streptavidin are covalently attached to per nanocrystals through an additional layer of polymer on them. [33–35]

In one of nano-chip based on signal transduction Quantum dots are attached to antibodies that gets attached to antigens which are captured on agarose beads array that is present inside a microfluidic assembly.[36]

In another detection system two types of arrays were compared one in which dye labelled anti -antibody was used and, in another Streptavidin, conjugated Quantum dots is used. Later one gives better performance.[37–39]

Using quantum dots in detection system ensures high degree of improvement in target detection, time of detection, sensitivity and reduction in assay cost. It also increases multiplexing capabilities. They offer large excitation coefficient, high photostability, low photobleaching, large stroke shift and inertness to pH change.[35,40–42]

High hepatotoxicity and poor solubility of Geldanamycin and other quinone based HSP inhibitors have prevented its clinical application as an anti-cancer agent, having a unique action of inhibiting HSP90. Drugs like 17-AAG have poor bioavailability.[5]

Niosomes are vesicles made up of hydrating mixture of non-ionic surfactant and cholesterol or other lipids. It is formed by self-assembly of surfactant molecules. It is used for delivering lipophilic and amphiphilic drugs. It increases stability and solubility of drugs. It provides targeted delivery and controlled release of compound. It also helps in crossing Blood-Brain barrier.[43–47]

In conventional method of making niosomes there is bulk mixing of aqueous and lipid phases. This process is not well controlled and is time consuming. It leads to large particles with high polydispersity causing dosage problem of niosomes. Using microfluidic channel bulk production of narrow sized niosomes can be achieved. It has rapid and controlled mixing of two phases in microchannels.[47,48]

Size of niosomes can be changed by changing flow rate and flow rate ratio of two phases. High flow rate ratio leads to bottom up synthesis and has strong correlation with size.[49,50]

During preparation of niosomes in microfluidic channel lipid is dissolved in organic phase which mixes with aqueous medium. Type of aqueous media has significant effect on characteristic of niosomes. It is also important for the drug which is to be encapsulated.[51,52]

3. Material and methods

3.1 Materials

3.1.1 Reagents

- Streptavidin coated Q-Dots -655 (Sigma Aldrich)
- 10X PBS Buffer
- Distilled water
- Blocking Buffer (6% BSA)-3g of BSA (Sigma Aldrich) is mixed in 5 mL 1X PBS and 45 mL distilled water
- Blocking Buffer (3% BSA)- 3g of BSA (Sigma Aldrich) is mixed in 5 mL 1X PBS and 45 mL distilled water
- Washing buffer- 1X PBS- 10 mL 10X PBS in 90 mL distilled water.
- Storage Buffer (1 L)- Tris HCl (pH 7.5)- 20 mM, KCl- 50 mM, β-Mercaptoetnanol- 6 mM, Glycerine- 10% (v/v), Distilled Water to make volume 1 L, sterile filtration.
- FP1 Buffer (10X, 50 mL)- Hepes- 20 mM, KCl-5 mM, Tween 20- 0.01%(v/v), BSA- 0.1 mg/mL, Distilled Water to make up the volume 50 mL.
- HSP 90 Buffer (1 L)- Tris HCl (pH 7.5)- 20 mM, KCl- 50 mM, β-Mercaptoetnanol- 6 mM, Glycerine- 10% (v/v), Distilled Water to make volume 1 L, sterile filtration.
- Biotin-ATP (Jena BioScience)
- Cy3-ATP (Jena BioScience)
- Cy5-ATP (Jena BioScience)
- Bacterial HSP
- Inhibitor Cocktail Bacteria (Protease)
- HeLa Cells
- Saliva
- Span60 (Avanti polar lipids)
- Cholesterol (Sigma Aldrich)
- PEG (Sigma Aldrich)
- Ethanol

3.1.2 Equipment

3.1.2.1 Nano-plotter

It is a non- contact micro arraying system that pipettes liquid in picolitre for high quality spotting. It uses piezoelectric pipetting tips made up of glass and silicon. Pipettes follow drop on demand principle, i.e., a drop is released only when the actuator is triggered. This leads to the bending of silicon diaphragm causing compression of fluid inside the pipette and finally drop is released.

Figure 5. GeSiM nanoplotter

3.1.2.1 NanoAssmblr Benchtop

Microfluidics has recently appeared as a new method of manufacturing liposomes, which lets reproducible mixing in milliseconds on the nanolitre scale. Due to smaller dimensions in a micromixer, there is fast mixing, dominated by diffusion or convection. Microfluidics refers to fluid handling methods in a controlled volume, typically below millimetre scales, which allows for the implementation of the mixing process into planar chips. Mixing profile permits for the stretching and folding of the fluid streams over the channel's cross-sectional area, which increases mass transfer.

NanoAssemblr system aids rapid, reproducible and scalable synthesis of homogeneous next-generation nanoparticles and liposomes. Lipid is dissolved in a solvent and it is pumped

into one inlet whereas aqueous buffer is pumped into the other inlet of the cartridge allowing microfluidic mixing. Nanoprecipitation reaction results in the formation of nanoparticles. This reaction takes place at the interface of the solvent and aqueous streams. Formation of Liposome is based on polarity alterations throughout the chamber and an increase in the surface area of the fluid interface occurs, when fluids fold on top of each other aided by the design and grooves on the channel floor.

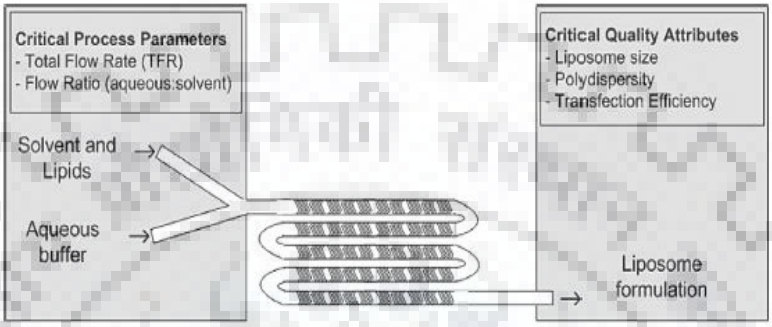


Figure 6. Microfluidic channel design

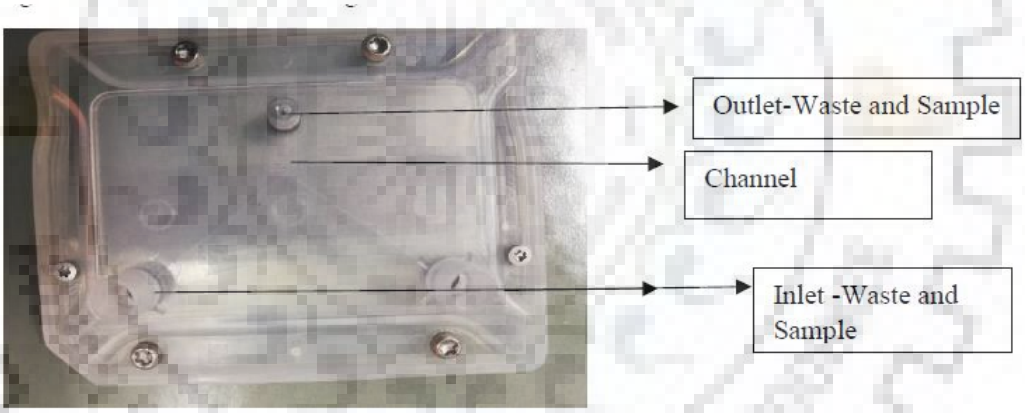


Figure 7. Microfluidic channel Cassette

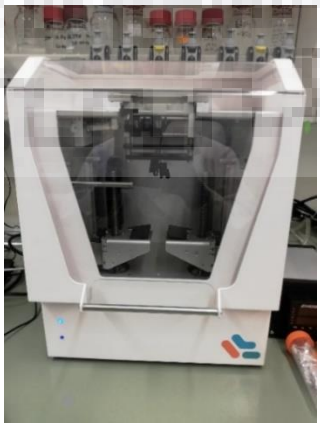


Figure 8. Nanoassmblr Benchtop

3.2 Methods

3.2.1 Miniaturization of Microarray based Screening of HSP inhibitors using nanoparticles

3.2.1.1 Preparing Q-Dots solutions of different concentration

- From the stock solution of the Streptavidin conjugated Q-dots solution of 1 µM concentration, 100 nM, 80 nM, 60 nM, 40 nM, 20 nM, 10 nM and 1 nM solutions are made. The dilution is carried out using 10X PBS buffer.
- 50 µL of each solution is transferred to 96 wells microwell plate.
- For storage microwell plate is covered with plastic film and Aluminium foil and stored at 4°C under dark condition.

3.2.1.2 Spotting

- These solutions are spotted on the microarray slide using Nano-Plotter. In each block, the concentration varied across a row and there are 10 spots of same concentration in one column.
- Column 1- 100 nM, Column 2- 80 nM, Column 3- 60 nM, Column 4- 40 nM, Column 5- 20 nM, Column 6- 10 nM, Column 7- 1 nM.
- After spotting is over, slide is kept for drying for 15 min at room temperature.
- Image of all the blocks is taken under Blue light for 10 ms, 20 ms and 40 ms exposure time. This is done to check the emission of different concentration of Q-Dots under different exposure time.

3.2.1.3 Blocking-

- 6% BSA and 3% BSA is used as locking buffer.
- Microarray slide is fixed in the incubation chamber. To each pad 150 µL of the blocking buffer is added. The chamber is covered with plastic slip.
- Incubation is carried out for 45 min on rotatory shaker at room temperature.

- Blocks are washed three times, with 1X PBS (washing buffer). 50 µL of the washing buffer is added to each pad, 5 min incubation is done on rotatory shaker at room temperature.
- Slide is removed from the incubation chamber.
- Blocks are completely dried then their image is taken under Blue light with 20 ms exposure time. This is done to check the effect of blocking buffer.

3.2.1.4 Adding of Protein-Biotin-ATP solution to the Q-Dots' spots

- Binder solution of Protein-Biotin-ATP is made from 11 mg/ mL $X_c$htpg and 5 mM Biotin-ATP stock solution.
- Stock solution of Biotin-ATP is thawed on ice for 45 min till the entire solution melted.
- 50 µL of 100 µM Biotin-ATP solution is made using 1X PBS and stock solution. This 100 µM Biotin-ATP is used for making binder solution of 10 and 50 µM Biotin-ATP with 3 mg/ mL $X_c$htpg(protein).
- Further dilutions of the binder solutions are carried out using storage buffer. All the binder solutions temperature is maintained around 4°C.
- Column 1 of the previous slide is blocked with 6% BSA.
- Three times washing is done with 1X PBS.
- 50 µL/ pad binder solution is added. 45 min incubation is done on rotatory shaker at room temperature.
- Three times washing is done with 1X PBS.
- Slide is removed from the chamber and dried at room temperature.
- Image is taken under blue light with 20 ms exposure time.

I. 10 µM Biotin-ATP	II. 50 µM Biotin-ATP
A. 3 mg/ mL protein- 20 µL stock protein, 6 µL 100 µM Biotin-ATP and 34 µL Storage buffer.	A. 3 mg/ mL protein- 20 µL stock protein, 30 µL 100 µM Biotin-ATP and 10 µL Storage buffer.

B. 300 µg/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.	B. 300 µg/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.
C. 30 µg/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.	C. 30 µg/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.
D. 3 µg/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.	D. 3 µg/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.
E. 300 ng/ mL protein- 6 µL from above solution and 54 µL of Storage buffer.	E. 300 ng/ mL protein- 6 µL from above solution and 54 µL of Storage buffer

Table 1. 10 µM and 50 µM Biotin-ATP solutions with 3mg/ mL-300 ng/ mL concentration of protein

	1	**2**
A	BLANK	BLANK
B	BLANK	BLANK
C	3 mg/ mL protein	3 mg/ mL protein
D	300 µg/ mL protein	300 µg/ mL protein
E	30 µg/ mL protein	30 µg/ mL protein
F	3 µg/ mL protein	3 µg/ mL protein
G	300 ng/ mL protein	300 ng/ mL protein
H	BLANK	BLANK

Table 2. Incubation Chamber with solutions of 3 mg/ mL-300 ng/ mL protein concentration

3.2.1.5 Masking of Q-Dots' signal by Biotin-ATP

- Biotin-ATP stock solution is thawed on ice till all the ice melted.
- Different solutions of 10 µM, 1 µM, 100 nM,10 nM, 1 nM, 100 pM and 10 pM are made from 5 mM stock solution. Dilution is carried out with 1X PBS.
- Solutions are incubated on ice for 1 hr.
- Q-Dots spotted on the microarray slide are blocked with 3% BSA. 100 µL of blocking buffer is added per pad, 45 min incubation is done on rotatory shaker at room temperature.
- Three times washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at room temperature.
- 50 µL of Biotin-ATP solution is added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Three times washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 45 min incubation is done on rotatory shaker at room temperature.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

500 µL of 10 µM Biotin-ATP- 1 µL of 5 mM stock+ 499 µL of 1X PBS
60 µL of 1 µM Biotin-ATP- 6 µL of 10 µM solution+ 54 µL 1X PBS
60 µL of 100 nM Biotin-ATP- 6 µL of 1 µM solution+ 54 µL 1X PBS
60 µL of 10 nM Biotin-ATP- 6 µL of 100 nM solution+ 54 µL 1X PBS
60 µL of 1 nM Biotin-ATP- 6 µL of 10 nM solution+ 54 µL 1X PBS
60 µL of 100 pM Biotin-ATP- 6 µL of 1 nM solution+ 54 µL 1X PBS
60 µL of 10 pM Biotin-ATP- 6 µL of 100 pM solution+ 54 µL 1X PBS

Table 3. Solutions of 1 µM- 10 pM Biotin-ATP concentration

	1	2
A	BLANK	BLANK
B	BLANK	BLANK
C	1 µM Biotin-ATP	1 µM Biotin-ATP
D	100 nM Biotin-ATP	100 nM Biotin-ATP
E	10 nM Biotin-ATP	10 nM Biotin-ATP
F	1 nM Biotin-ATP	1 nM Biotin-ATP
G	100 pM Biotin-ATP	100 pM Biotin-ATP
H	10 pM Biotin-ATP	10 pM Biotin-ATP

Table 4. Incubation Chamber with solutions of 1 µM- 10 pM Biotin-ATP concentration

3.2.1.6 Masking of Q-Dots' signal by Protein-Biotin-ATP

- 11 mg/ mL (157.14 µM) protein ($X_c$htpg) and 5 mM Biotin-ATP stocks are taken.
- 90 µL from protein stock and 5.4 µL from Biotin-ATP stock are mixed together and incubated on ice for 1hr. Protein concentration reduces to 141.5 µM and Biotin-ATP reduces to 3.53 mM.
- 1:10 dilution is carried out with FP1 buffer.
- Solution is added to PD-30 column and centrifuged thrice at 3500g, 4°C for 15 min each.
- Protein concentration becomes 150 µM.
- Dilution of protein-ATP binder solution is carried out with FP1 buffer and Storage buffer.

180 µL of 50 µM Protein concentration- 60 µL of 150 µM protein+ 120 µL of 1X FP1

160 µL of 5.6 µM Protein concentration- 18 µL of 50 µM protein+ 120 µL of 1X FP1+22 µL of Storage Buffer
160 µL of 0.6 µM Protein concentration- 18 µL of 5.6 µM protein+ 120 µL of 1X FP1+22 µL of Storage Buffer
160 µL of 70 nM Protein concentration- 18 µL of 0.6 µM protein+ 120 µL of 1X FP1+22 µL of Storage Buffer
160 µL of 7.89 nM Protein concentration- 18 µL of 70 nM protein+ 120 µL of 1X FP1+22 µL of Storage Buffer

Table 5. Protein-Biotin-ATP solutions of 50 µM-70 nM concentration

- Blocking and washing is done in the previous way.
- 50 µL of Binder solution is added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Washing is done.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1	2
A	BLANK	BLANK
B	50 µM Protein	50 µM Protein
C	5.6 µM Protein	5.6 µM Protein
D	0.6 µM Protein	0.6 µM Protein
E	70 nM Protein	70 nM Protein
F	7.9 nM Protein	7.9 nM Protein
G	50 µM Protein	5 µM Protein

H	0.6 µM Protein	70 nM Protein

Table 6. Incubation Chamber with solutions of 50 µM-70 nM Protein-Biotin-ATP concentration

3.2.1.7 Masking of Q-Dots' signal by Protein-Biotin-ATP

- 460 µL of 3 mg/ mL (42.86 µM) protein ($X_c$htpg) is taken.
- 395 µL volume of protein solution required to be to make concentration 50 µM.
- Filtration of protein is carried out with PD30 column by centrifuged at 3500g, 4°C for 15 min.
- 165 µL of Storage buffer is added to above protein and the concentration becomes 50 µM.
- To the above solution 3.95 µL of 5 mM Biotin-ATP is added. Biotin-ATP concentration in solution is 50 µM. To remove unbound ATP filtration of the above solution is done with PD30 column by five times centrifugation at 3500g, 4°C for 15 min each.

Ist round centrifugation	395 µL protein + 4 mL Storage buffer	Volume reduces to 290 µL
IInd round centrifugation	290 µL protein + 4 mL Storage buffer	Volume reduces to 200 µL
IIIrd round centrifugation	200 µL protein + 4 mL Storage buffer	Volume reduces to 180 µL
IVth round centrifugation	180 µL protein + 4 mL Storage buffer	Volume reduces to 160 µL
Vth round centrifugation	160 µL protein + 4 mL Storage buffer	Volume reduces to 160 µL

Table 7. Protein and storage buffer used for different rounds of filtration through PD30 column

- Dilution of the filtered solution is carried out with Storage buffer.

60 µL of 5 µM Protein Concentration- 6 µL of 50 µM filtered protein solution+ 54 µL of Storage buffer.

50 µL of 1 µM Protein Concentration- 1.2 µL of 50 µM filtered protein solution+ 48.8 µL of Storage buffer
60 µL of 500 nM Protein Concentration- 6 µL of 5 µM protein solution+ 54 µL of Storage buffer
60 µL of 50 nM Protein Concentration- 6 µL of 500 nM protein solution+ 54 µL of Storage buffer
60 µL of 5 nM Protein Concentration- 6 µL of 50 nM protein solution+ 54 µL of Storage buffer
60 µL of 500 pM Protein Concentration- 6 µL of 5 nM protein solution+ 54 µL of Storage buffer
60 µL of 50 pM Protein Concentration- 6 µL of 500 pM protein solution+ 54 µL of Storage buffer.

Table 8. Filtered Protein-Biotin-ATP solutions of 5 µM-50 pM concentration

- Blocking and washing is done.
- 50 µL of Binder solution is added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Three times washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at room temperature.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1	2
A	BLANK	BLANK
B	5 µM Protein	5 µM Protein
C	1 µM Protein	1 µM Protein
D	500 nM Protein	500 nM Protein
E	50 nM Protein	50 nM Protein
F	5 nM Protein	5 nM Protein
G	500 pM Protein	500 pM Protein
H	50 pM Protein	50 pM Protein

Table 9. Incubation Chamber with solutions of 5 µM-50 pM filtered Protein-Biotin-ATP concentration

3.2.1.8 Masking of Q-Dots' signal by Protein-Biotin-ATP

- 3mg/ mL (42.86 µM) protein ($X_c$htpg) is taken.
- Filtration of protein is carried out with PD30 column by centrifuged at 3500g, 4°C for 15 min. After which volume reduced to 230 µL.
- Storage buffer is added to above protein and the concentration becomes 50 µM.
- To the above solution 3.95 µL of 5 mM Biotin-ATP is added. Biotin-ATP concentration in solution is 50 µM.
- To remove unbound ATP filtration of the above solution is done with PD30 column by five times centrifugation at 3500g, 4°C for 15min each.

Ist round centrifugation	400 µL protein + 5 mL Storage buffer
IInd round centrifugation	5 mL Storage buffer added

IIrd round centrifugation	4 mL Storage buffer added
IVth round centrifugation	4 mL Storage buffer added
Vth round centrifugation	4 mL Storage buffer added

Table 10. Protein and storage buffer used for different rounds of filtration

- Dilution of the filtered solution is carried out with Storage buffer.

60 µL of 1 nM Protein Concentration- 6 µL of 10 nM filtered protein solution+ 54 µL of Storage buffer.
60 µL of 100 pM Protein Concentration- 6 µL of 1 nM filtered protein solution+ 48.8 µL of Storage buffer
60 µL of 10 pM Protein Concentration 6 µL of 100 pM protein solution+ 54 µL of Storage buffer
60 µL of 1 pM Protein Concentration- 6 µL of 10M protein solution+ 54 µL of Storage buffer
60 µL of 100 fM Protein Concentration- 6 µL of 1 pM protein solution+ 54 µL of Storage buffer
60 µL of 10 fM Protein Concentration- 6 µL of 100 fM protein solution+ 54 µL of Storage buffer
60 µL of 1 fM Protein Concentration- 6 µL of 10 fM protein solution+ 54 µL of Storage buffer.
60 µL of 100 oM Protein Concentration- 6 µL of 1 fM protein solution+ 54 µL of Storage buffer.
60 µL of 10 oM Protein Concentration- 6 µL of 100 oM protein solution+ 54 µL of Storage buffer.

Table 11. Filtered Protein-Biotin-ATP solutions of 1 nM-10 oM concentration

- Blocking and washing is done.
- 50 µL of Binder solution is added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Washing is done.
- Microarray slide is dried at room temperature.

- Image is taken under Blue light for 20 ms exposure.

	1	2
A	1 nM Protein	1 nM Protein
B	100 pM Protein	100 pM Protein
C	10 pM Protein	10 pM Protein
D	1 pM Protein	1 pM Protein
E	100 fM Protein	100 fM Protein
F	1 fM Protein	1 fM Protein
G	100 oM Protein	100 oM Protein
H	10 oM Protein	10 oM Protein

Table 12. Incubation Chamber with solutions of filtered Protein-Biotin-ATP of 1 nM-10 oM concentration

3.2.1.9 Checking masking of Q-Dots' signal by Protein-Biotin-ATP with and without inhibitor

- Concentration of Stock protein ($X_c$htpg) is 13.6 mg/ mL i.e. 194.21 µM. Inhibitor used is Radicicol and its concentration is 2.7 mM.
- Two solutions A and B are made with 50 µM Protein and Biotin-ATP concentration. One is with inhibitor and other one is without inhibitor.

Solution A- without inhibitor	Solution B- With inhibitor
Protein- 25.8 µL from stock	Protein- 25.8 µL from stock
Biotin-ATP- 1 µL from 5 mM stock	Biotin-ATP- 1 µL from 5 mM stock
Inhibitor- 0	Inhibitor- 1.85 µL from stock

1X PBS- 73.2 µL	1X PBS- 71.35 µL

Table 13. Protein-Biotin-ATP solutions with and without inhibitor

- Both solutions are incubated on ice for 2 h.
- Solutions are filtered through PD-10 column (gravity filtration) at room temperature.
- After filtration around 3.4 mL of both solutions are obtained.
- Solutions are concentrated through PD-30 column by centrifugation at 3500g, 4°C for 15 min.
- 45 µL of solution A and 52 µL of solution B is obtained. Concentration of protein in both solutions are obtained using Nanodrop. Solution A has 81.42 µM and solution B has 48.97 µM.
- Solution A is diluted with 1X PBS so that its concentration is same as that of B.
- Solutions are diluted to different concentration with 1X PBS.

60 µL of 5 µM Protein Concentration- 6.12 µL of 48.97 µM filtered protein solution+ 53.88 µL of Storage buffer.
60 µL of 500 nM Protein Concentration- 6 µL of 5 µM filtered protein solution+ 54 µL of Storage buffer
60 µL of 50 nM Protein Concentration- 6 µL of 500 nM protein solution+ 54 µL of Storage buffer
60 µL of 5 nM Protein Concentration- 6 µL of 50 nM protein solution+ 54 µL of Storage buffer
60 µL of 500 pM Protein Concentration- 6 µL of 5 nM protein solution+ 54 µL of Storage buffer
60 µL of 50 pM Protein Concentration- 6 µL of 500 pM protein solution+ 54 µL of Storage buffer
60 µL of 5 pM Protein Concentration- 6 µL of 50 pM protein solution+54 µL of Storage buffer.

Table 14. Protein-Biotin-ATP solutions of 1 nM-10oM concentration with and without inhibitor

- Blocking and washing is done.
- 50 µL of Storage buffer is added to one of the pads to check its effect on Q-Dots.

- 50 µL of Binder solution is added to each pad. 45min incubation is done on rotatory shaker at room temperature.
- Washing is done.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms and 30 ms exposure.

	1(Solution A without inhibitor)	2(Solution B with inhibitor)
A	1 nM Protein	1 nM Protein
B	100 pM Protein	100 pM Protein
C	10 pM Protein	10 pM Protein
D	1 pM Protein	1 pM Protein
E	100 fM Protein	100 fM Protein
F	1 fM Protein	1 fM Protein
G	100 oM Protein	100 oM Protein
H	10 oM Protein	10 oM Protein

Table 15. Incubation Chamber with solutions of filtered Protein-Biotin-ATP of 1 nM-10 oM concentration with and without inhibitor

3.2.1.10 Checking protein activity with Cy3 labelled ATP

- Concentration of stock protein ($X_c$htpg) is 13.6 mg/ mL i.e. 194.28 µM. It is diluted to 3 mg/ mL i.e. 42.85 µM with chilled Storage buffer. Temperature is maintained 4°C.
- 50 µL of the protein is transferred to microwell plate and it is spotted on the nitrocellulose slide. Slide is air dried for 15 min.

- Blocking is done with 1% BSA in HSP90 buffer. 100 µL/pad blocking buffer is added and incubated for 45 min on shaker at room temperature.
- Stock Cy3-ATP is of 1 mM concentration and it is diluted to 10 µM with distilled water.
- A negative control of 100 nM Cy3-ATP and FP1 buffer is made.
- Different inhibitors (HK581F10, HK571, Radicicol, HK592, HK571d, HK56b) of unknown concentration are added to 100 nM Cy3-ATP and FP1buffer solution. Inhibitors are diluted ten-fold in the solutions and hundred-fold diluted inhibitor solution of Radicicol and HK581F10 are also made.
- After blocking slide is washed three times with HSP90 buffer, with 5 min incubation time each, on rotatory shaker at room temperature.
- 50 µL/pad of inhibitor solution and negative control is added to the slide. Hybridization chamber is covered with Aluminium foil and kept in a box laid with wet tissue papers at 4°C for overnight incubation.
- Next day, three times washing was done with chilled FP1 buffer, with 5 min incubation time each, on rotatory shaker at 4°C.
- Slide is dried and image is taken under Green light with 50 ms exposure time.

	1	2
A	Negative Control	Negative Control
B	HK581F10	HK581F10
C	HK571	HK571
D	RADICICOL	RADICICOL
E	HK592	HK592
F	HK571d	HK571d
G	HK56b	HK56b
H	RADICICOL (100X)	HK581F10 (100X)

Table 16. Incubation Chamber with different HSP inhibitors

3.2.1.11 Checking protein activity with Cy5 labelled ATP

- Concentration of stock protein ($X_c$htpg) is 13.6 mg/ mL i.e. 194.28 µM. It is diluted to 3 mg/ mL i.e. 42.85 µM with chilled Storage buffer. Temperature is maintained 4°C.
- 50 µL of the protein is transferred to microwell plate and it is spotted on the nitrocellulose slide. Slide is air dried for 15 min.
- Blocking is done with 1% BSA in HSP90 buffer. 100 µL/pad blocking buffer is added and incubated for 45 min on shaker at room temperature.
- After blocking slide is washed three times with HSP90 buffer, with 5 min incubation time each, on rotatory shaker at room temperature.
- Stock Cy5-ATP is of 1 mM concentration and it is diluted to 10 µM with distilled water.
- A negative control of 100 nM Cy5-ATP and FP1 buffer is made.
- Different inhibitors (HK581F10, HK571, Radicicol, HK592, HK571d, HK56b) of unknown concentration are added to 100 nM Cy5-ATP and FP1buffer solution. Inhibitors are diluted ten-fold in the solutions and hundred-fold diluted inhibitor solution of Radicicol and HK581F10 are also made.
- 50 µL/pad of inhibitor solution and negative control is added to the slide. Hybridization chamber is covered with Aluminium foil and kept in a box laid with wet tissue papers at 4°C for overnight incubation.
- Next day, three times washing was done with chilled FP1 buffer, with 5 min incubation time each, on rotatory shaker at 4°C.
- Slide is dried and image is taken under Green light with 50 ms exposure time.

	1	2
A	Negative Control	Negative Control
B	HK581F10	HK581F10
C	HK571	HK571
D	RADICICOL	RADICICOL

E	HK592	HK592
F	HK571d	HK571d
G	HK56b	HK56b
H	RADICICOL (100X)	HK581F10 (100X)

Table 17. Incubation Chamber with different HSP inhibitors

3.2.1.12 Checking masking of Q-Dots' signal by Protein-Biotin-ATP with & without inhibitor

- Stock protein concentration is 13.6mg/ mL, Biotin-ATP is 1 µM and unknown concentration of Inhibitor (Radicicol).
- Solutions A and B without and with inhibitor are made respectively. Concentration of protein reduces to 3 mg/ mL i.e. 42.85, Biotin-ATP reduces to 100 nM and inhibitor is diluted ten-fold.

Solution A- without inhibitor	Solution B- With inhibitor
Protein- 22.5 µL from stock	Protein- 22.5 µL from stock
Biotin-ATP- 10 µL from stock	Biotin-ATP- 10 µL from stock
Inhibitor- 0	Inhibitor- 10 µL from stock
FP1- 68 µL	FP1- 58 µL

Table 18. Protein-Biotin-ATP solutions with and without inhibitor

- Overnight incubation is carried out at 4°C.
- Filtration of each solution is done separately with PD-30 column to remove unbound ATP. 100 µL of the solution with 3.9 mL 1X PBS is added to the filter and centrifuged at 3500g for 15 min at 4°C.
- Filtered solutions are serially diluted to different concentration of 4.2 µM, 0.42 µM and 0.042 µM with 1X PBS.

- Q-Dots spotted on the microarray slide are blocked with 3% BSA. 100 µL of blocking buffer is added per pad, 45 min incubation is done on rotatory shaker at room temperature.
- Three times washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at room temperature.
- 50 µL of solutions A and B are added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Three times washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at 4°C.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1(Solution A without inhibitor)	2(Solution B with inhibitor)
A	42 µM Protein	42 µM Protein
B	4.2 µM Protein	4.2 µM Protein
C	0.42 µM Protein	0.42 µM Protein
D	0.042 µM Protein	0.042 µM Protein
E	42 µM Protein	42 µM Protein
F	4.2 µM Protein	4.2 µM Protein
G	0.42 µM Protein	0.42 µM Protein
H	0.042 µM Protein	0.042 µM Protein

Table 19. Incubation Chamber with solutions of filtered Protein-Biotin-ATP of 42 µM-0.042 µM concentration with and without inhibitor

3.2.1.13 Checking masking of Q-Dots' signal by Protein-Biotin-ATP with & without inhibitor-

- Stock protein concentration is 13.6mg/ mL, Biotin-ATP is 10 µM and unknown concentration of inhibitor (Radicicol).
- Solutions A and B without and with inhibitor are made respectively. Concentration of protein reduces to 3 mg/ mL i.e. 42.85 µM, Biotin-ATP reduces to 100 nM and inhibitor is diluted ten-fold.
- In solution B inhibitor is added first and incubated for 1.5 h then Biotin-ATP is added.

Solution A- without inhibitor	Solution B- With inhibitor
Protein- 44 µL from stock	Protein- 44 µL from stock
Biotin-ATP- 2 µL from stock	Biotin-ATP- 2 µL from stock
Inhibitor- 0	Inhibitor- 20 µL from stock
FP1- 154 µL	FP1- 134 µL

Table 20. Protein-Biotin-ATP solutions with and without inhibitor

- Overnight incubation is carried out at 4°C.
- Filtration of each solution is done separately with PD-30 column to remove unbound ATP. 100 µL of the solution with 3.9 mL 1X PBS is added to the filter and centrifuged at 3500g for 15 min at 4°C.
- Filtered solutions are serially diluted to different concentration of 4.2 µM, 0.42 µM and 0.042 µM with 1X PBS.
- Blocking and washing is done.
- 50 µL of solutions A and B are added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Once washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at 4°C.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.
- Twice washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at 4°C.
- Microarray slide is dried at room temperature.

- Image is taken under Blue light for 20 ms exposure.

	1(Solution B with inhibitor)	2(Solution A without inhibitor)
A	Blank	Blank
B	42 µM Protein	42 µM Protein
C	4.2 µM Protein	4.2 µM Protein
D	0.42 µM Protein	0.42 µM Protein
E	0.042 µM Protein	0.042 µM Protein
F	4.2 µM Protein	4.2 µM Protein
G	0.42 µM Protein	0.42 µM Protein
H	0.042 µM Protein	0.042 µM Protein

Table 21. Incubation Chamber with solutions of filtered Protein-Biotin-ATP of 42 µM-0.042 µM concentration with and without inhibitor

3.2.1.14 Checking masking of Q-Dots' signal by inhibitor (Radicicol)

- Concentration of stock solution of Radicicol is 1 mg/ mL i.e. 2.74 mM
- Concentration is reduced to 10 µM with 10% DMSO.
- It is further diluted to 1 µM and 100 nM with 1X PBS.
- Blocking and washing is done.
- 50 µL of solutions A and B are added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Washing is done at 4°C.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1	2
A	Blank	Blank
B	1 µM Radicicol	1 µM Radicicol
C	100 nM Radicicol	100 nM Radicicol

Table 22. Incubation Chamber with 1 µM-100 nM concentration of Radicicol

3.2.1.15 Checking masking of Q-Dots' signal by Protein-Biotin-ATP with & without inhibitor

- Stock protein concentration is 13.6mg/ mL, Biotin-ATP is 10 µM and 10 µM concentration of inhibitor (Radicicol).
- Solutions A and B without and with inhibitor are made respectively. Concentration of protein reduces to 3 mg/ mL i.e. 42.85, Biotin-ATP reduces to 100 nM and inhibitor is diluted ten-fold.
- In solution B inhibitor is added first and incubated for 1.5 h then Biotin-ATP is added. Concentration of inhibitor becomes 100 nM.

Solution A- without inhibitor	Solution B- With inhibitor
Protein- 22 µL from stock	Protein- 22 µL from stock
Biotin-ATP- 1 µL from stock	Biotin-ATP- 1 µL from stock
Inhibitor- 0	Inhibitor- 1 µL from stock
FP1- 77 µL	FP1- 58 µL

Table 23. Protein-Biotin-ATP solutions with and without inhibitor

- Overnight incubation is carried out at 4°C.
- Filtration of each solution is done separately with PD-30 column to remove unbound ATP. 100 µL of the solution with 3.9 mL 1X PBS is added to the filter and centrifuged at 3500g for 15 min at 4°C.

- Filtered solutions are serially diluted to different concentration of 4.2 µM, 0.42 µM and 0.042 µM with 1X PBS.
- Blocking and washing is done.
- 50 µL of solutions A and B are added to each pad. 45 min incubation is done on rotatory shaker at room temperature.
- Once washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at 4°C.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1(Solution A without inhibitor)	2(Solution B with inhibitor)
A	42 µM Protein	42 µM Protein
B	4.2 µM Protein	4.2 µM Protein
C	0.42 µM Protein	0.42 µM Protein
D	0.042 µM Protein	0.042 µM Protein
E	Blank	Blank
F	Blank	Blank
G	Blank	Blank
H	Blank	Blank

Table 24. Incubation Chamber with solutions of filtered Protein-Biotin-ATP of 42 µM-0.042 µM concentration with and without inhibitor

3.2.1.16 Checking Masking of Q-dots' signal by Hela cells with and without inhibitor

- Frozen HeLa cells are thawed on ice for an hour.
- They are transferred to chilled 2 mL vials and centrifuged at 300g for 5 min at 4°C.
- 10 µL of Inhibitor Cocktail Bacteria (Protease) is added.
- To 950 µL twice 1.9 mL of 1X FP1 buffer is added.
- The above solution is passed through syringe eight times, to break open the cells.
- Solution is divided into two equal parts and centrifuged at 12000g for 10 min at 4°C.
- Pellet is discarded. 50 µL of the supernatant is taken for Western blot and rest for making with and without inhibitor solutions.
- In solution B inhibitor is added first and incubated for 1.5 h, then ATP is added. Inhibitor concentration in the solution becomes 100 nM.
- Overnight incubation is done 4°C after everything is added.
- Solution A is made with 99 µL. Filtration is done with PD30 column to remove unbound ATP. 100 µL of the solution with 3.9 mL 1X PBS is added to the filter and centrifuged at 3500g for 15 min at 4°C.
- Filtered sample is serially diluted six times with 1X PBS.
- Blocking and washing is done.
- 50 µL of solutions A and B are added to each pad. 45 min incubation done on rotatory shaker at room temperature.
- Once washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5min incubation is done on rotatory shaker at 4°C.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1(Solution A without inhibitor)	2(Solution B with inhibitor)
A	Blank	Blank
B	Sample	Sample
C	1X dilution	1X dilution
D	2X dilution	2X dilution
E	3X dilution	3X dilution
F	4X dilution	4X dilution
G	5X dilution	5X dilution
H	6X dilution	6X dilution

Table 25. Incubation Chamber with solutions of Hela cell lysate-Biotin-ATP of different concentration with and without inhibitor

3.2.1.17 Checking Masking of Q-dots' signal by Saliva with and without inhibitor

- Saliva sample is collected and diluted ten times with 1X PBS.
- Solutions A and B are with and without inhibitor are prepared.
- In solution B inhibitor is added first and incubated for 1.5 h, then ATP is added. Inhibitor concentration in the solution becomes 100 nM.
- Overnight incubation is done 4°C after everything is added.

Solution A- without inhibitor	Solution B- With inhibitor
Saliva- 99 µL	Saliva- 98 µL
Biotin-ATP- 1 µL from 10 µM stock	Biotin-ATP- 1 µL from 10 µM stock
Inhibitor- 0	Inhibitor- 1 µLfrom 10 µM stock

Table 26. Saliva-Biotin-ATP solutions with and without inhibitor

- Filtration is done with PD30 column to remove unbound ATP. 100 µL of the solution with 3.9 mL 1X PBS is added to the filter and centrifuged at 3500g for 15 min at 4°C.
- Filtered sample is serially diluted six times with 1X PBS.
- Blocking and washing is done.
- 50 µL of solutions A and B are added to each pad. 45 min incubation done on rotatory shaker at room temperature.
- Once washing is done with 1X PBS. 100 µL of washing buffer is added per pad, 5 min incubation is done on rotatory shaker at 4°C.
- Microarray slide is dried at room temperature.
- Image is taken under Blue light for 20 ms exposure.

	1(Solution A without inhibitor)	2(Solution B with inhibitor)
A	Blank	Blank
B	Sample	Sample
C	1X dilution	1X dilution
D	2X dilution	2X dilution
E	3X dilution	3X dilution
F	4X dilution	4X dilution
G	5X dilution	5X dilution
H	6X dilution	6X dilution

Table 27. Incubation Chamber with solutions of Saliva-Biotin-ATP of different concentration with and without inhibitor

3.2.1.18 Checking Stability of spotted Q-Dots

- Image of the spotted Q-Dots are taken under Blue light for 20 ms exposure.
- Side B of the slide is blocked with 3% BSA. 100 µL of blocking buffer per pad, 45 min incubation on shaker at room temperature.
- Slide is washed three times with 1X PBS. 100 µL of washing buffer per pad, 5 min incubation on shaker at room temperature.
- Slide is dried and image is taken under Blue light for 20 ms.
- Slide is stored at 4°C. and images are taken over a period.
- Diluted Quantum dots of 100 nM, 80 nM, 60 nM and 40 nM concentration is stored in microwell plate at 4°C under dark conditions. Spotting is done using these solutions over a period of 1.5 to 2.5 months.

3.2.2 Synthesising nanocarriers for inhibitors

3.2.2.1 Preparing Lipid Solution for Test I

- 0.0021 g of Span60, 0.0019 g of Cholesterol and 0.0003 g of PEG is measured. To Span60 and Cholesterol 400 µL of Distilled water is added and 200 µL to PEG.
- For proper mixing and breaking of large particles solutions are vortexed and sonicated for two minutes followed by mixing in the thermal mixer for one hour at 65°C.
- Four more same lipid solutions are made.

3.2.2.2 Formation of Niosomes for Test I

- Cassette is fitted in the Nanoassmblr and priming of the cassette is done with Ethanol and Distilled water before making niosomes.
- Following values are entered in the software-

  Volume= 2 mL

  Flow rate ratio (F.R) = 1:1

  Total flow rate (TFR) = 12 mL/min

  Left syringe size = 3 mL, Right syringe size= 1 mL

Start waste volume= 0.035 mL, End waste volume= 0.005 mL

- To make niosomes Distilled water and lipid solution is taken in 1 mL syringes. Distilled water syringe is fitted in the left inlet of cassette and Lipid solution in the right inlet.
- Temperature is maintained at 65°C using heating block around the syringe.
- Following values are entered in the software-

  Volume= 1.2 mL

  Flow rate ratio (F.R) = 1:1

  Total flow rate (TFR)= 12 mL/min

  Left syringe size = 1 mL, Right syringe size= 1 mL

  Start waste volume= 0.035 mL, End waste volume= 0.005 mL
- Niosomes are formed.
- Overnight Dialysis is carried out to remove Ethanol.
- Zeta sizer is used to measure the size, PdI and Zeta potential of the Niosomes.
- To make different sized niosomes different flow rate ratio is adjusted.

Sample	Volume(mL)	F.R	Aqueous syringe dispense(mL)	Lipid syringe dispense(mL)	TFR(mL/min)
Sa	1.2	1:1	0.6	0.6	4
Sb	1.2	2:1	0.8	0.4	4
Sc	1.2	3:1	0.9	0.3	4
Sd	1.2	4:1	0.96	0.24	4
Se	1.2	5:1	1.0	0.2	4

Table 28. Different flow rate ratio for test 1 niosomes

Figure 9. Preparation of Niosomes **Figure 10.** Dialysis

3.2.2.3 Preparing Lipid Solution for Test II

- The concentration of lipid components is doubled.
- 0.0042 g of Span60, 0.0038 g of Cholesterol and 0.0006 g of PEG is measured.
- For proper mixing and breaking of large particles solutions are vortexed and sonicated for two minutes followed by mixing in the thermal mixer for one hour at 65°C.
- Four more same lipid solutions are made.

3.2.2.4 Formation of Niosomes for Test II

- Cassette is fitted in the Nanoassmblr and priming of the cassette is done with Ethanol and Distilled water before making niosomes.
- Following values are entered in the software-

  Volume= 2 mL

  Flow rate ratio (F.R) = 1:1

  Total flow rate (TFR)= 12 mL/min

  Left syringe size = 3 mL, Right syringe size= 1 mL

  Start waste volume= 0.035 mL, End waste volume= 0.005 mL

- To make niosomes Distilled water and lipid solution is taken in 1 mL syringes. Distilled water syringe is fitted in the left inlet of cassette and Lipid solution in the right inlet.
- Temperature is maintained at 65°C using heating block around the syringe.
- Following values are entered in the software-

  Volume= 2 mL

  Flow rate ratio (F.R) = 1:1

  Total flow rate (TFR) = 4 mL/min

  Left syringe size = 1 mL, Right syringe size= 1 mL

  Start waste volume= 0.035 mL, End waste volume= 0.005 mL
- Niosomes are formed.
- Overnight Dialysis is carried out to remove Ethanol.
- Zeta sizer is used to measure the size, PdI and Zeta potential of the Niosomes.
- To make different sized niosomes different flow rate ratio is adjusted.

Sample	Volume(mL)	F.R	Aq. syringe dispense(mL)	Lipid syringe dispense(mL)	TFR(mL/min)
Sa	2	1:1	1.0	1.0	4
Sb	2	2:1	1.33	0.67	4
Sc	2	3:1	1.50	0.50	4
Sd	2	4:1	1.60	0.40	4
Se	2	5:1	1.67	0.33	4

Table 29. Different flow rate ratios for test 2 niosomes

3.2.2.5 Preparing Lipid Solution for Test III

- The concentration of lipid components is doubled.
- 0.0042 g of Span60, 0.0038 g of Cholesterol and 0.0006 g of PEG is measured.

- For proper mixing and breaking of large particles solutions are vortexed and sonicated for two minutes followed by mixing in the thermal mixer for one hour at 65°C.
- Four more same lipid solutions are made.
- After making 1 mL of lipid solution 20 µL of FITC labelled 17AAG of different concentration is added to different solutions.

Sample	Drug	100% DMSO	Concentration
Sa	20 µL from stock	0	1 mM
Sb	3 µL from stock	27 µL	1 µM
Sc	3 µL from stock	27 µL	1 µM
Sd	2 µL from Sb	18 µL	1 nM
Se	2 µL from Sc	18 µL	1 nM

Table 30. Different concentration of drug

3.2.2.6 Formation of Niosomes for Test III

- Cassette is fitted in the Nanoassmblr and priming of the cassette is done with Ethanol and Distilled water before making niosomes.
- Following values are entered in the software-

  Volume= 2 mL

  Flow rate ratio (F.R) = 1:1

  Total flow rate (TFR) = 12 mL/min

  Left syringe size = 3 mL, Right syringe size= 1 mL

  Start waste volume= 0.035 mL, End waste volume= 0.005 mL
- To make niosomes Distilled water and lipid solution is taken in 1 mL syringes. Distilled water syringe is fitted in the left inlet of cassette and Lipid solution in the right inlet.
- Temperature is maintained at 65°C using heating block around the syringe.
- Following values are entered in the software-

Volume= 2 mL

Flow rate ratio (F.R) = 1:1

Total flow rate (TFR) = 4 mL/min

Left syringe size = 1 mL, Right syringe size= 1 mL

Start waste volume= 0.035 mL, End waste volume= 0.005 mL

- Niosomes are formed.
- Overnight Dialysis is carried out to remove Ethanol.
- Zeta sizer is used to measure the size, PdI and Zeta potential of the Niosomes.
- Flow rate ratio is kept same for all the samples.

Sample	Volume(mL)	F.R	Aqueous syringe dispense(mL)	Lipid syringe dispense(mL)	TFR(mL/min)
Sa	2	1:1	1.0	1.0	4
Sb	2	1:1	1.0	1.0	4
Sc	2	1:1	1.0	1.0	4
Sd	2	1:1	1.0	1.0	4
Se	2	1:1	1.0	1.0	4

Table 31. Flow rate ratios of niosomes loaded with drug

4. Results and discussions

4.1 Miniaturization of Microarray based Screening of HSP inhibitors using nanoparticles

4.1.1 Spotting

- Different concentration of Q-Dots is spotted and different exposure time for blue light is used.

Figure 11. 10 ms exposure time

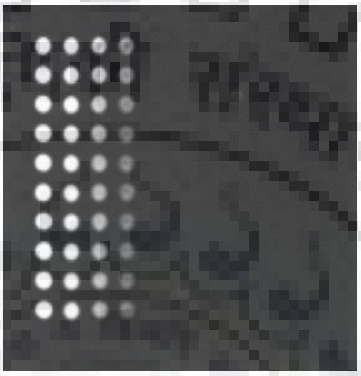


Figure 12. 20 ms exposure time

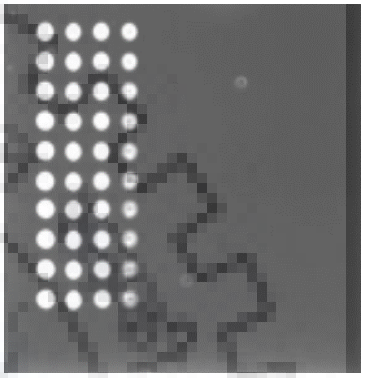


Figure 13. 40 ms exposure time

- Under 10, 20 and 40 ms only 100, 80, 60 and 40 nM concentration of Q-Dots spots are visible.
- This might be due to lower concentration of Q-Dots' spots aren't able to capture enough energy for fluorescence.
- Out of the three-exposure time in case of 10 ms only 100 nM concentration has higher brightness rest all have low brightness.
- In 40 ms all the different concentration spots have almost the same level of brightness.
- For 20 ms there is a gradual decrease in brightness from 100-40 nM concentration.
- Thus, 20 ms exposure suits best for irradiating Quantum Dots.

4.1.2 Effects of blocking buffer

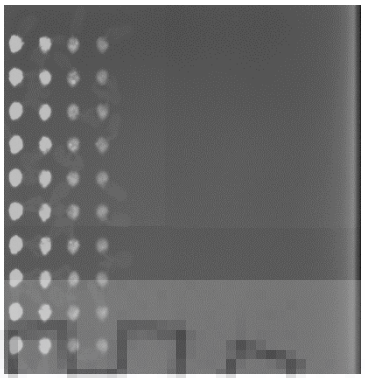
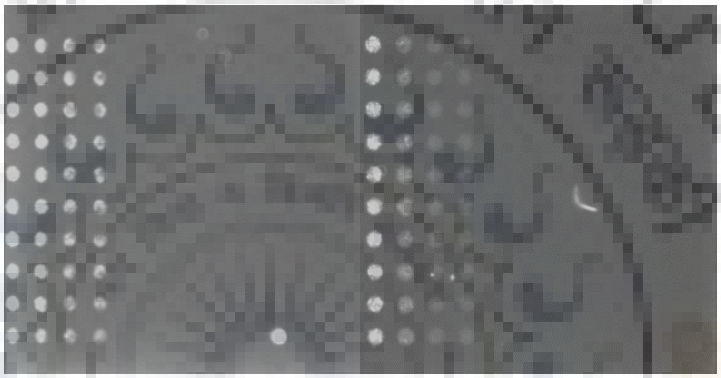


Figure 14. Without Blocking buffer



3% BSA 6% BSA

Figure 15. After adding Blocking buffer

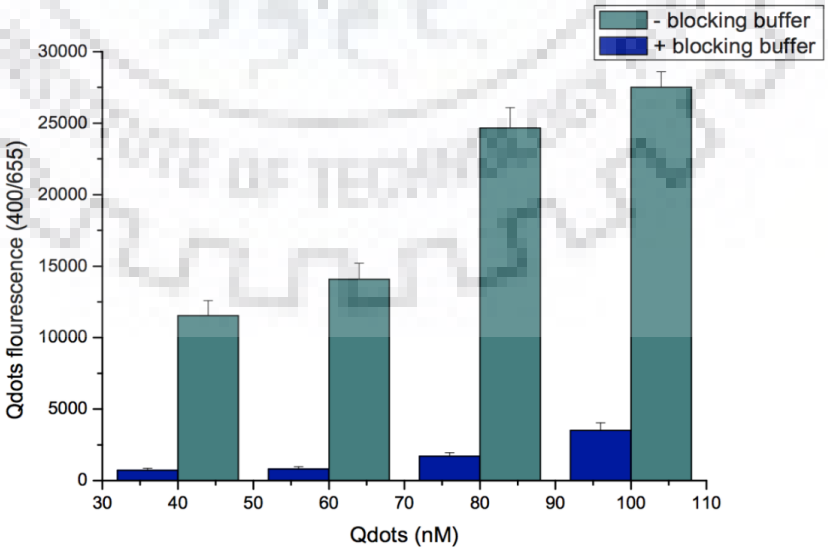


Figure 16. Graph showing decrease in Q-Dots' signal after blocking with 6% BSA

- Blocking buffer didn't completely mask signal from Q-Dot spots. Although the brightness of 60 and 40 nM concentration decreased around 90%.
- For 100 nM and 80 nM decrease in signal is around 80 and 85%. As the concentration of Q-Dots increases the decrease in signal is less due to blocking is less.

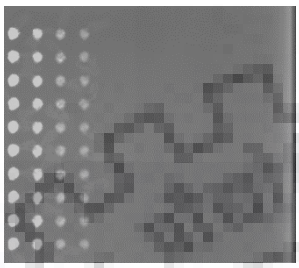


Figure 17. No blocking

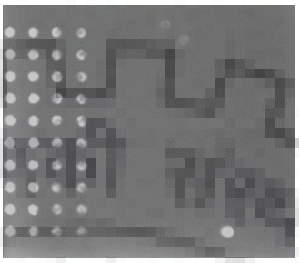


Figure 18. 3% BSA

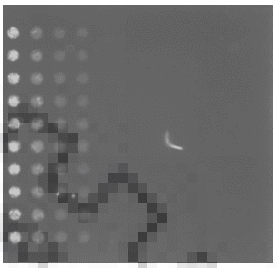


Figure 19. 6% BSA

- When compared 3% BSA blocking buffer is better than 6% BSA as the decrease in signal is much lesser for all the different concentrations.

4.1.3 Adding Protein-Biotin-ATP to the Q-Dots' spots

- No spots are observed for the pads in which binder solution is added. In blank spots with diminished brightness are observed due to addition of blocking buffer.

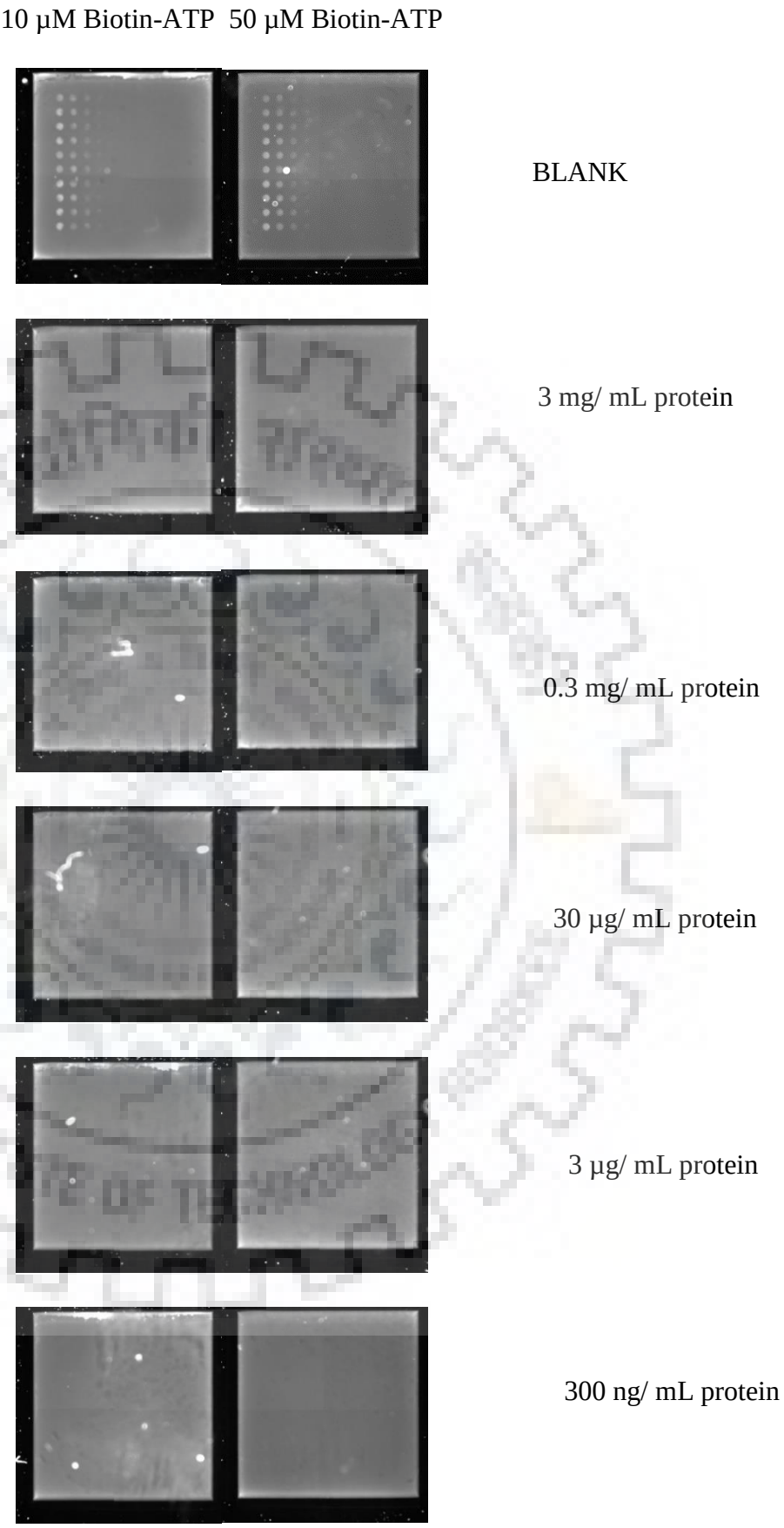


Figure 20. Microarray slide with Protein-Biotin-ATP

- Maybe concentration of protein or free Biotin-ATP is too high that they block all the signal.

4.1.4 Masking of Q-dots' signal by Biotin-ATP

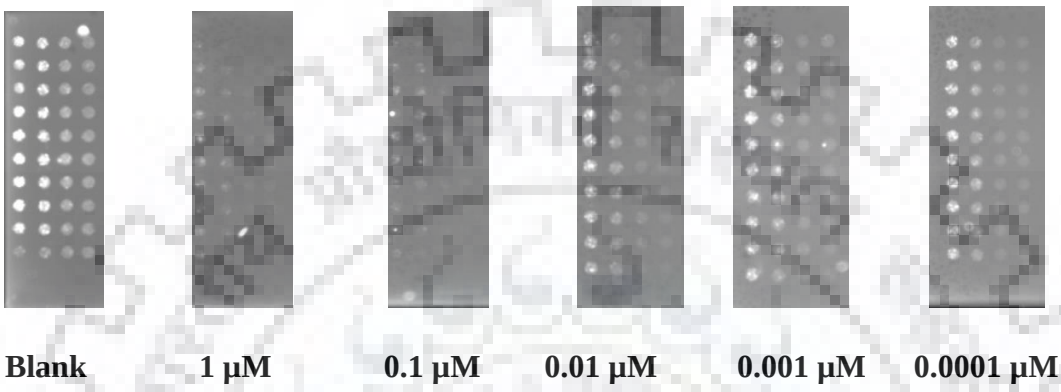


Figure 21. Microarray slide with different concentration of Biotin-ATP solutions added

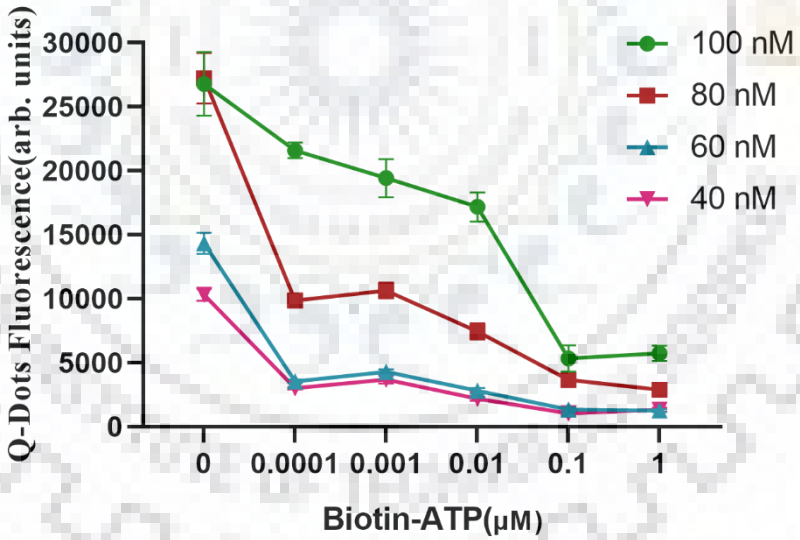


Figure 22. Graph showing change in fluorescence of Q-Dots' spots of different concentration after adding Biotin-ATP

- The detection range of the Q-Dot's microarray when Biotin ATP is added lies between 100 nM- 1 nM concentration of Biotin-ATP for 100 nM and 80 nM Q-Dot concentration.

- This is represented by steep region of the dose-response curve. Concentration above 100 nM and below 1 nM lie in the saturation range.
- For above 100 nM concentration of Biotin-ATP is too high that most of the signal is blocked and much change isn't observed with change in concentration.
- Below 1 nM concentration is too less to significantly mask Q-Dots' fluorescence. Hence, saturation is seen below 1 nM.

4.1.5 Masking of Q-dots' signal by Protein- Biotin-ATP

- For 50 µM- 5 nM range none of the Quantum dots spots are not visible on the microarray slide. This might be due to high concentration of protein or unbound ATP.
- Concentration should be reduced to picomolar range.

4.1.6 Masking of Q-dots' signal by Protein- Biotin-ATP

- For 5 µM- 50 pM range none of the Quantum dots spots are not visible on the microarray slide. This might be due to high concentration of protein.
- Maybe the sensitivity is too high, and the detection range lies in octa molar concentration of protein.

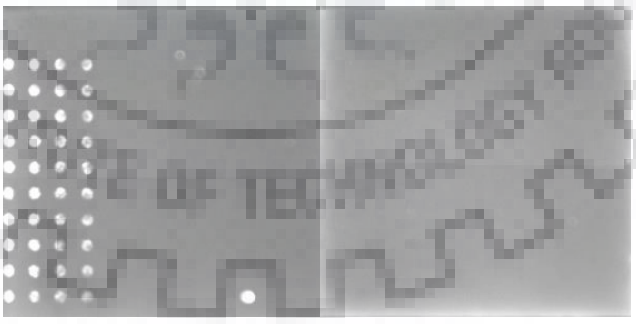


Figure 23. No buffer **Figure 24**. After adding Protein-Biotin-ATP

4.1.7 Masking of Q-dots' signal by Protein- Biotin-ATP

- For 1 nM- 10oM range none of the Quantum dots spots are visible on the microarray slide.

- Maybe the storage buffer is causing bleaching of the Quantum dots' fluorescence.

4.1.8 Checking Masking of Q-dots' signal by Protein- Biotin-ATP with and without inhibitor

- Nothing is observed on the storage buffer pad. Storage buffer causes photobleaching of Quantum dots.

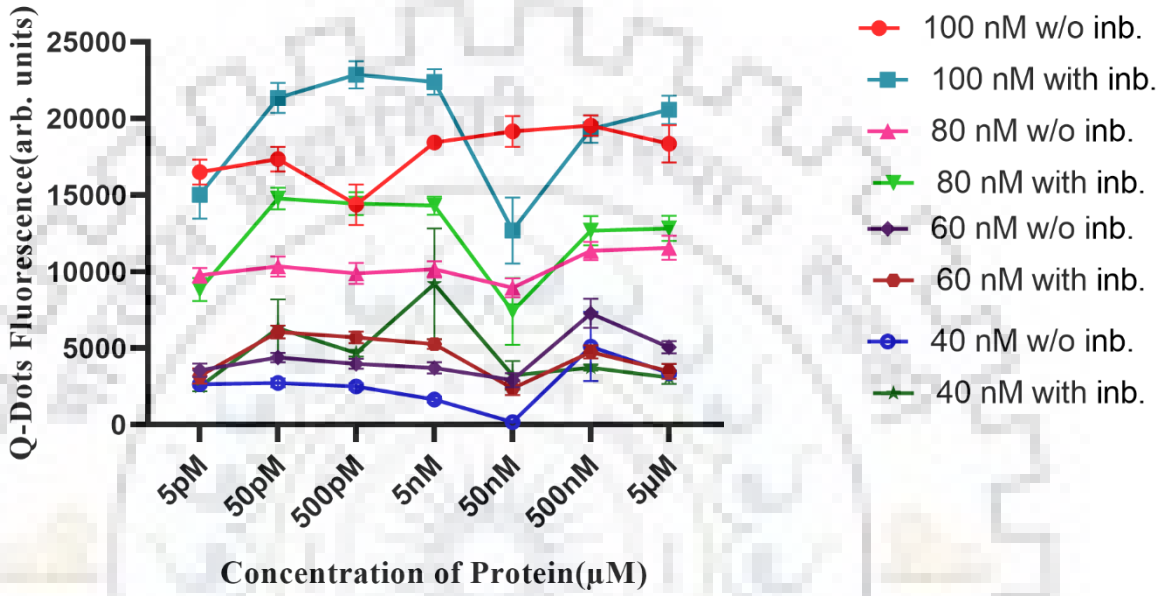


Figure 25. Graph showing change in fluorescence of Q-Dots' spots after adding different concentration of Protein-Biotin-ATP solutions without inhibitor (w/o inb.) and with inhibitor (with inb.)

- For 100 nM Q-Dots' spots 5 µM, 5 nM, 500 pM, 50 pM with inhibitor gives less masking effect than without inhibitor solution. For 500 nM, 50 nM and 5 pM it is vice versa.
- For 80 nM Q-Dots' spots 5 µM, 500 nM, 5 nM, 500 pM and 50 pM with inhibitor gives less masking effect than without inhibitor solution. For 50 nM and 5 pM it is opposite.
- For 60 nM Q-Dots' spots 5 nM, 500 pM and 50 pM with inhibitor gives less masking effect than without inhibitor solution. For 5 µM, 500 nM, 50 nM and 5 pM it is opposite.
- For 80 nM Q-Dots' spots 50 nM, 5 nM, 500 pM and 50 pM with inhibitor gives less masking effect than without inhibitor solution. For 5 µM, 500 nM and 5 pM it is opposite.
- For without Inhibitor pads there is uneven decrease in fluorescence of Q-Dots' spots.
- For with inhibitors there is increase in signal for 100 nM spots from 50 nM to 50 pM concentration range. Almost same signal for 5 µM and 500 nM. Same is with 80 nM spots. For 60 nM and 40 nM spots there is uneven change in signal.

- In case of without inhibitor protein solutions there should be decrease in signal from 100 nM spots to 40 nM spots.
- In case of with inhibitors solutions there shouldn't be much change in the signal along the decrease in protein concentration as protein isn't bound to Biotin-ATP hence it does not mask the signal from Q-Dots.
- Q-Dots to which without inhibitor solutions are added should give less signal compared to the ones with inhibitors. This is seen in only in some cases.
- This is happening because protein might be inactive. Gel filtration with PD-10 column might cause inactivation of protein as the protein during this step is at room temperature for a long time. If it is not inactive then it requires more time to incubate with ATP and inhibitor. FP1 buffer should be used for filtration and incubation.

4.1.9 Checking Protein activity with Cy3 labelled ATP

- Bright spots are seen for Negative control (Cy3-ATP+FP1).
- There is very high background. This can be reduced by changing Cy3-ATP with Cy5-ATP.
- In case of HK581F10 Inhibitor slightly bright spots are seen. This shows it is inhibiting attachment of Cy3 labelled ATP with HSP to certain extend.
- Highly bright spots are seen in case of HK571. This is not a good inhibitor.
- Even for Radicicol bright spots are seen. It is not able to inhibit the attachment of Cy3-ATP with the Heat shock protein.
- No spots are seen for HK592 compound. This shows it is a very good inhibitor.
- For HK592 bright spots are seen. It is not inhibiting.
- Even HK571d isn't inhibiting.

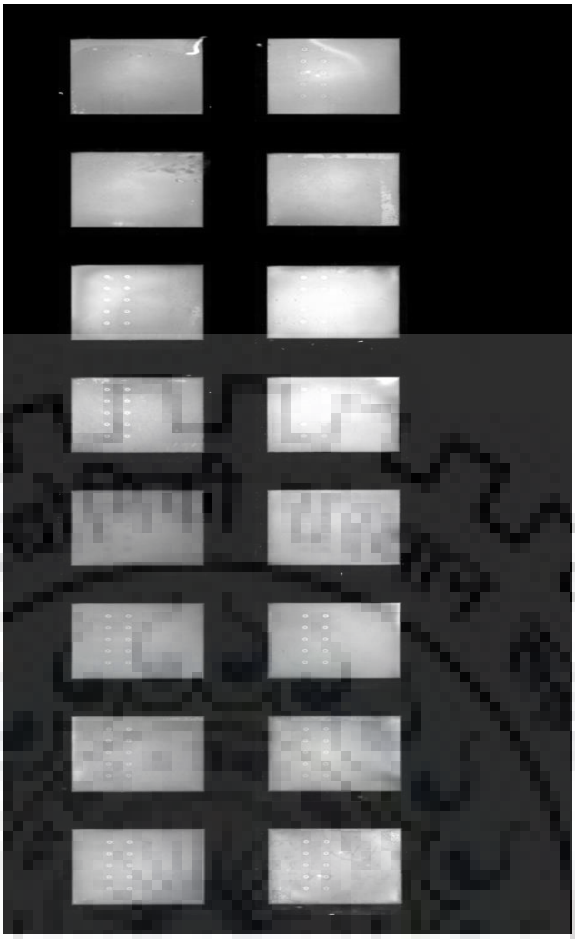


Figure 26. Microarray slide with Protein spots with Cy3-ATP and different inhibitors

4.1.10 Checking Protein activity with Cy5 labelled ATP

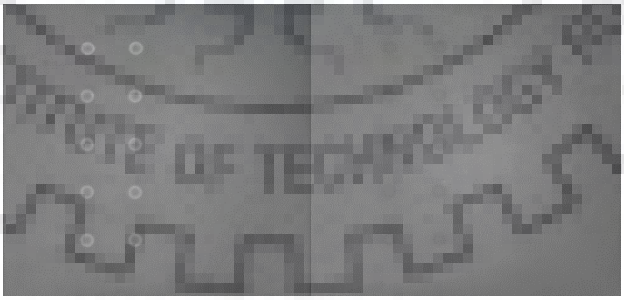


Figure 27. Showing with Cy5-ATP (control) and with Cy5-ATP and Radicicol

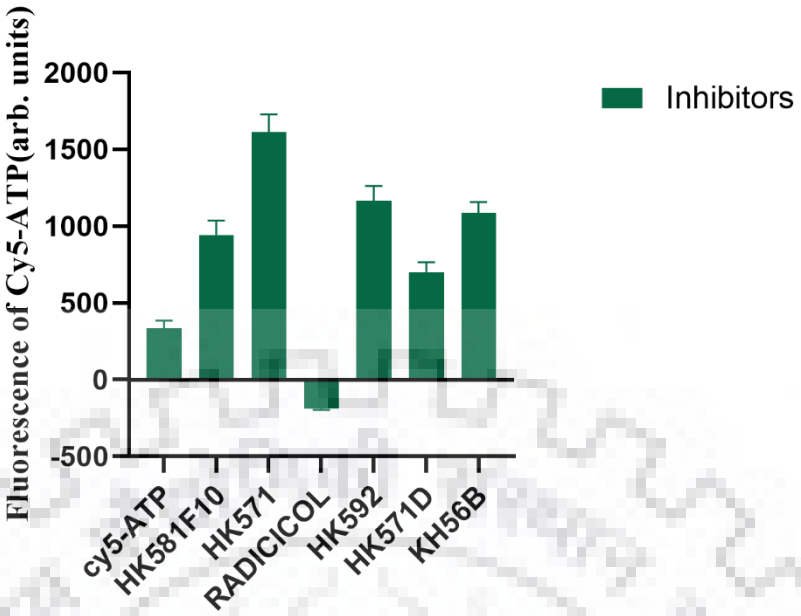


Figure 28. Graph showing fluorescence of Cy5 labelled ATP attached to spotted Heat shock protein in presence of inhibitors

- Protein is active as it is bright spots in negative control.
- Radicicol completely inhibits attachment of Cy5 labelled ATP with the protein.
- There is some extent of inhibition in case of HK581F10 and HK571d.
- Background noise is less in this case compared to Cy3 label.

4.1.11 Checking Masking of Q-dots' signal by Protein- Biotin-ATP with and without inhibitor

- Most of the without Inhibitor solutions are showing less masking of Q-Dots' fluorescence compared to with Inhibitor solutions.
- Opposite of what is expected is seen. It might be happening because inhibitor gets attached ATP bound to protein, in a way that ATP will remain attached to protein instead of getting displaced.
- First Inhibitor should be added and incubated with protein then ATP should be added.

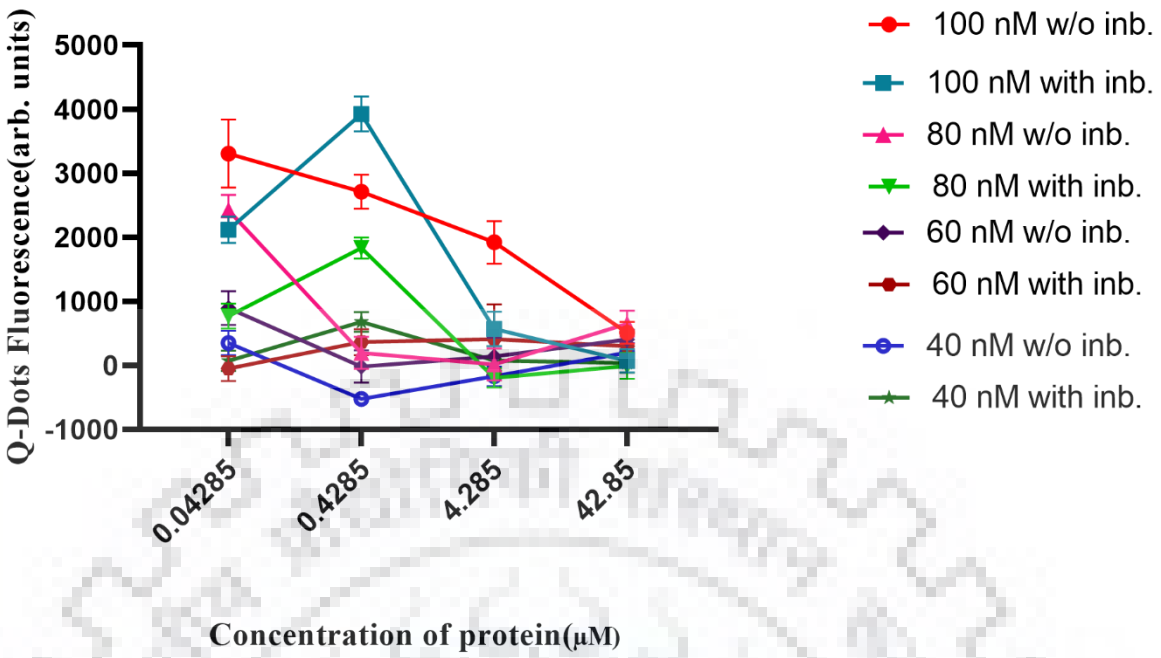


Figure 29. Graph showing change in fluorescence of Q-Dots' spots after adding different concentration of Protein-Biotin-ATP solutions without inhibitor (w/o inb.) and with inhibitor (with inb.)

4.1.12 Checking Masking of Q-dots' signal by Protein- Biotin-ATP with and without inhibitor

- Only for 42 µM without inhibitor solution masking of fluorescence of 100 nM Q-Dots' spots was more compared to with inhibitor solution.
- For 42 µM and 0.42 µM without inhibitor solution masking of fluorescence of 80 nM Q-Dots' spots was more compared to with inhibitor solution.
- For 4.2 µM, 0.42 µM and 0.042 µM without inhibitor solution masking of fluorescence of 60 nM Q-Dots' spots was more compared to with inhibitor solution.
- For all without inhibitor solutions masking of fluorescence of 40 nM Q-Dots' spots was more compared to with inhibitor solution.
- Inhibitor concentration is too high. It is masking a lot of signal. The concentration needs to be optimized.

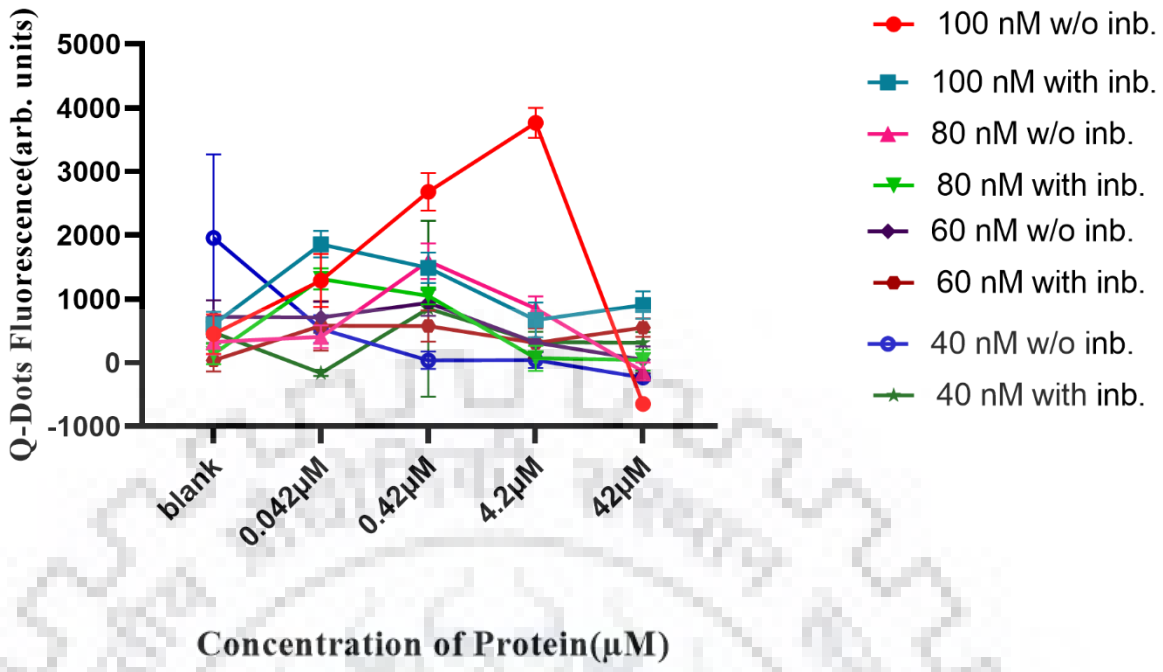


Figure 30. Graph showing change in fluorescence of Q-Dots' spots after adding different concentration of Protein-Biotin-ATP solutions without inhibitor (w/o inb.) and with inhibitor (with inb.)

4.1.13 Checking Masking of Q-dots' signal by inhibitor (Radicicol)

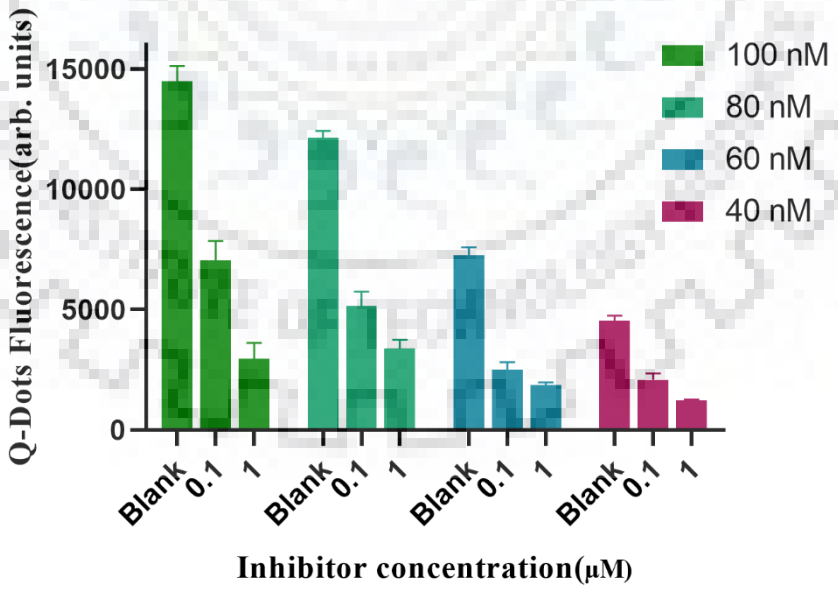


Figure 31. Bar Graph showing change in fluorescence of Q-Dots' spots after adding different concentration of inhibitor (Radicicol)

- 1 µM and 100 nM Radicicol showed reduction in signal. For 100 nM reduction isn't very high compared to 1 µM concentration.
- Reduction in signal is happening due to unspecific binding of Radicicol with Streptavidin by hydrophobic interactions.
- 100 nM Radicicol can be used for further experiments.

4.1.14 Checking Masking of Q-dots' signal by Protein- Biotin-ATP with and without inhibitor

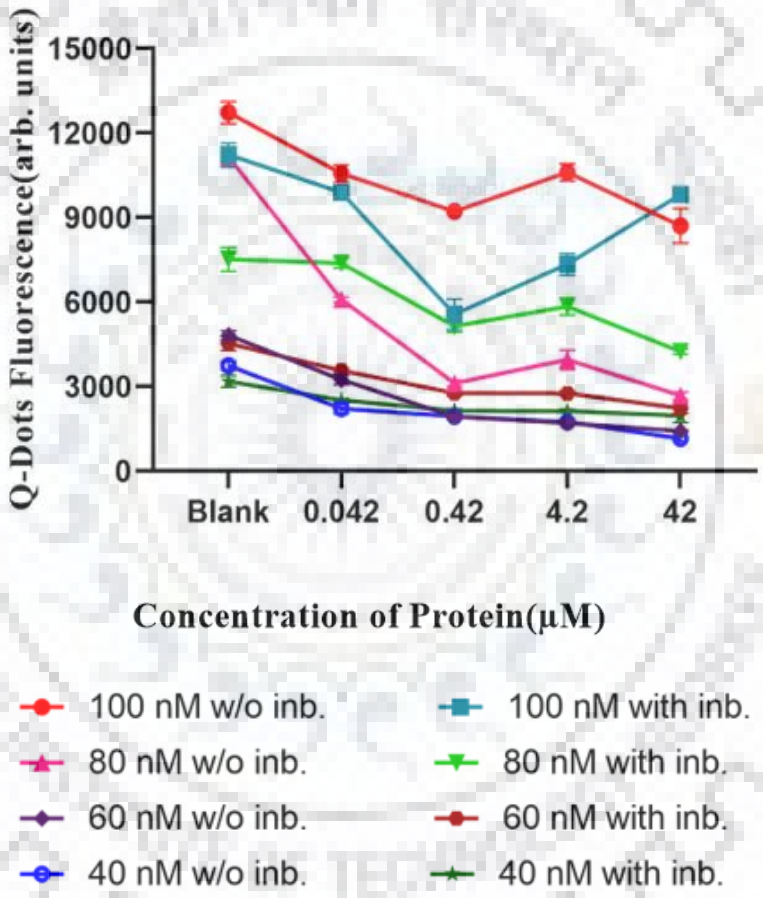


Figure 32. Graph showing change in fluorescence of Q-Dots' spots after adding solutions of different concentration of Protein-Biotin-ATP without inhibitor (w/o inb.) and with inhibitor (with inb.)

- For 100 nM, 80 nM, 60 nM and 40 nM Q-Dots' spots in case of blank pads little difference in fluorescence is seen between without and with inhibitor solutions pads. This is due decrease in the fluorescence of the spots due to long storage time of the microarray slide.

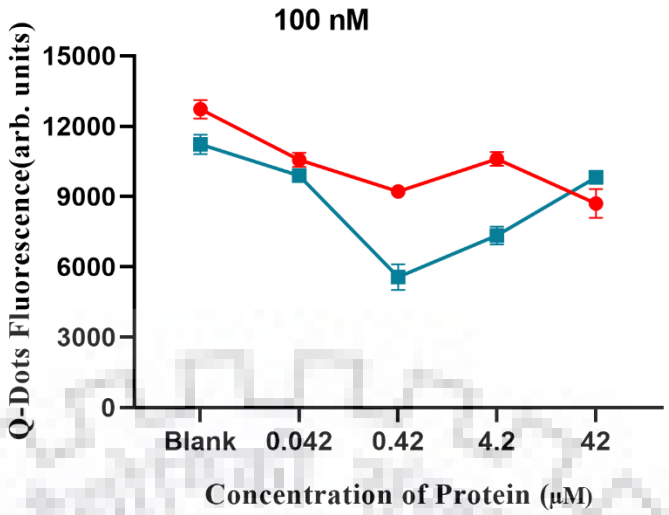


Figure 33. Graph showing change in fluorescence of Q-Dots' spots of 100 nM concentration after adding solutions of different concentration of Protein-Biotin-ATP without (w/o inb.) and with inhibitor (with inb.)

- For 100 nM spots masking of fluorescence by with inhibitor solution is more than without inhibitor solution for all the concentration except 42 µM
- Basic principle of this Quantum Dot Microarray is not working. Maybe 100 nM spots are too bright to be for lower concentration to block the fluorescence properly or more molecules of inhibitors can attach to the 100 nM concentration spots thus making more fluorescence compared to the without inhibitor solution.

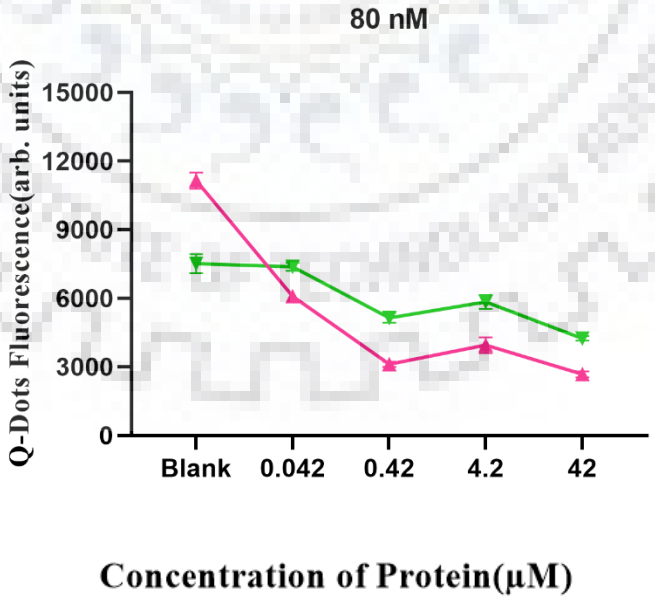


Figure 34. Graph showing change in fluorescence of Q-Dots' spots of 80 nM concentration after adding solutions of different concentration of Protein-Biotin-ATP without (w/o inb.) and with inhibitor (with inb.)

- For 80 nM Q-Dots' spots principle is working for the given protein concentrations. The difference with and without inhibitor is sufficiently good. There is decrease in fluorescence with increase in protein concentration except at 4.2 µM.

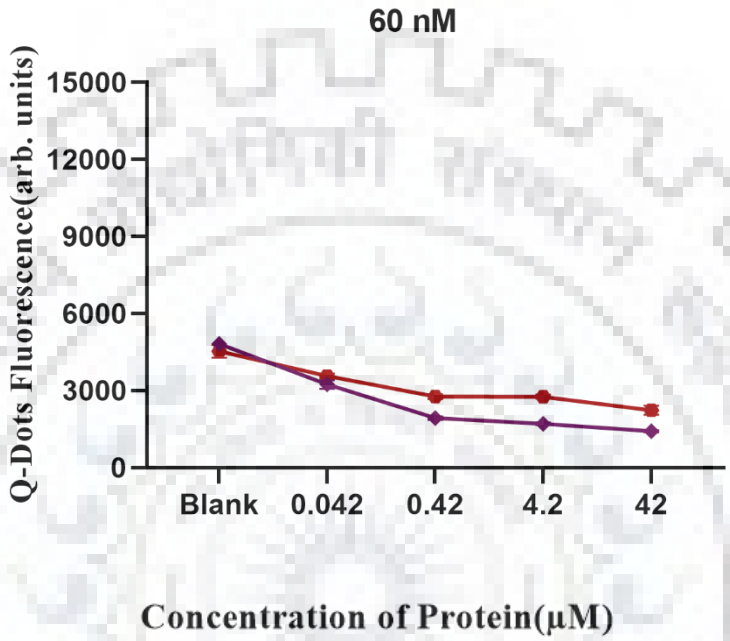


Figure 35. Graph showing change in fluorescence of Q-Dots' spots of 60 nM concentration after adding solutions of different concentration of Protein-Biotin-ATP without (w/o inb.) and with inhibitor (with inb.)

- For 60 nM Q-Dots' spots principle is working for the given protein concentrations. The difference with and without inhibitor is less compared to 80 nM. There is decrease in fluorescence with increase in protein concentration.

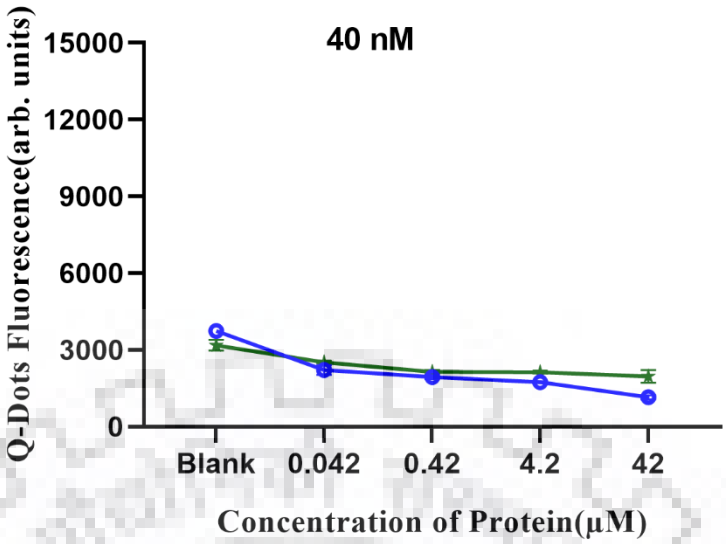


Figure 36. Graph showing change in fluorescence of Q-Dots' spots of 40 nM concentration after adding solutions of different concentration of Protein-Biotin-ATP without Inhibitor and with Inhibitor

- For 40 nM Q-Dots' spots principle is working for the given protein concentrations. The difference with and without inhibitor is very less compared to 80 nM, points are close to each other. There is decrease in fluorescence with increase in protein concentration.
- 80 nM and 60 nM concentration of Quantum dots suits best for the array. A better concentration dependent graph with saturation regions can be obtained if more points are included in between 42 µM-4.2 µM and 0.42 µM to blank range.

4.1.15 Checking Masking of Q-dots' signal by Hela cells with and without inhibitor

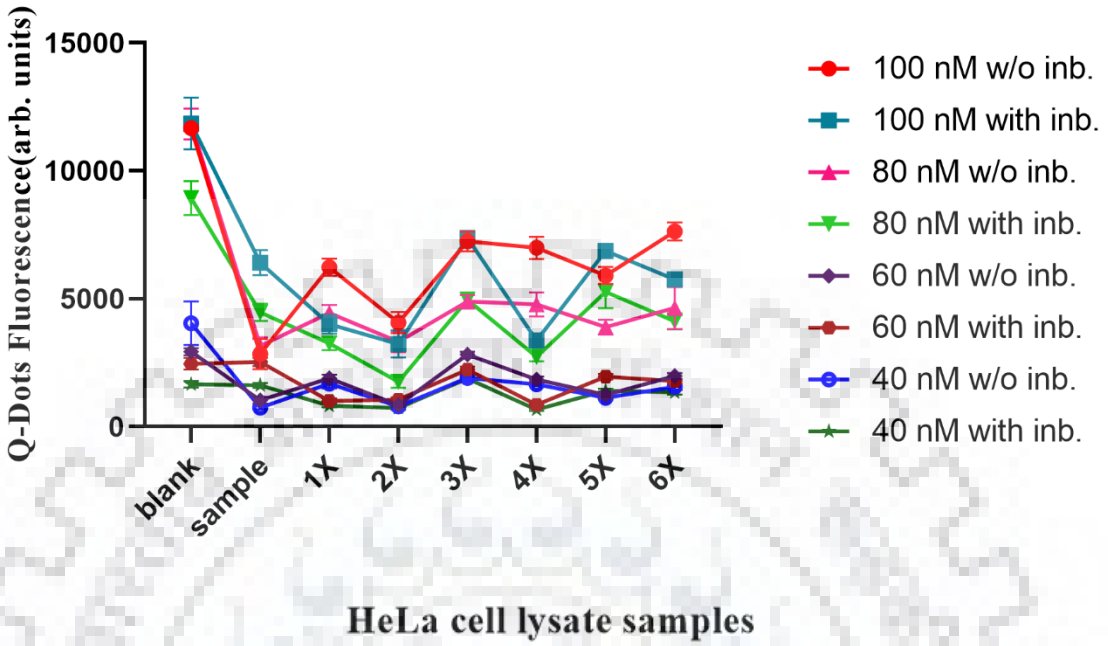


Figure 37. Graph showing change in difference in fluorescence of Q-Dots' spots of after adding solutions of different dilutions of HeLa cell lysate without inhibitor (w/o inb.) and with inhibitor (with inb.). 1X – 2X are dilution steps

- Heat shock protein is detected in the cell lysate of HeLa cells.
- For sample with no dilution the difference in fluorescence of with and without inhibitor is positive and for 1X to 6X dilutions difference is negative.

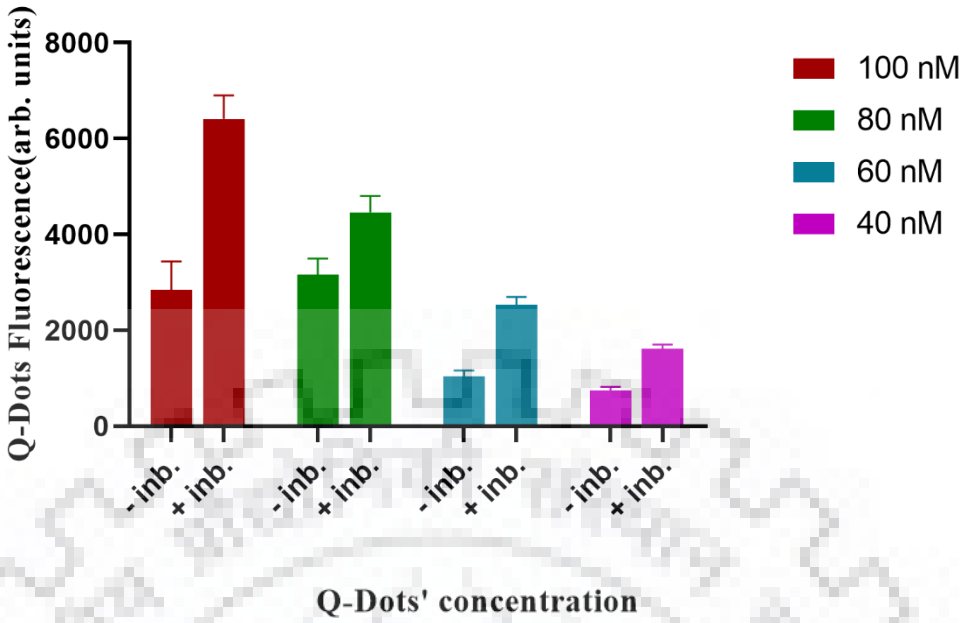


Figure 38. Bar graph showing change in difference in fluorescence of Q-Dots' spots after adding Hela cell lysate solutions without dilution with inhibitor (+inb.) and without inhibitor (-inb.)

- This shows in case of Sample with inhibitor cell lysate Heat shock Protein can't bind with Biotin-ATP which in turn does not bind to Streptavidin coated Quantum dots. With inhibitor cell lysate has lesser masking effect than without Inhibitor.
- No further dilution of the cell lysate is required.
- Western blot confirms the presence of Heat Shock protein in the cell lysate of HeLa cells.
- A big band is seen in lane 1 near 110kDa. That is HSP90 with post translational phosphorylation.
- Another band is seen around 140kDa. This is the dimer of HSP70. Dimer is seen as sample isn't heated properly before loading into the gel.
- Smudge is seen due degradation of HSP90 and HSP70.

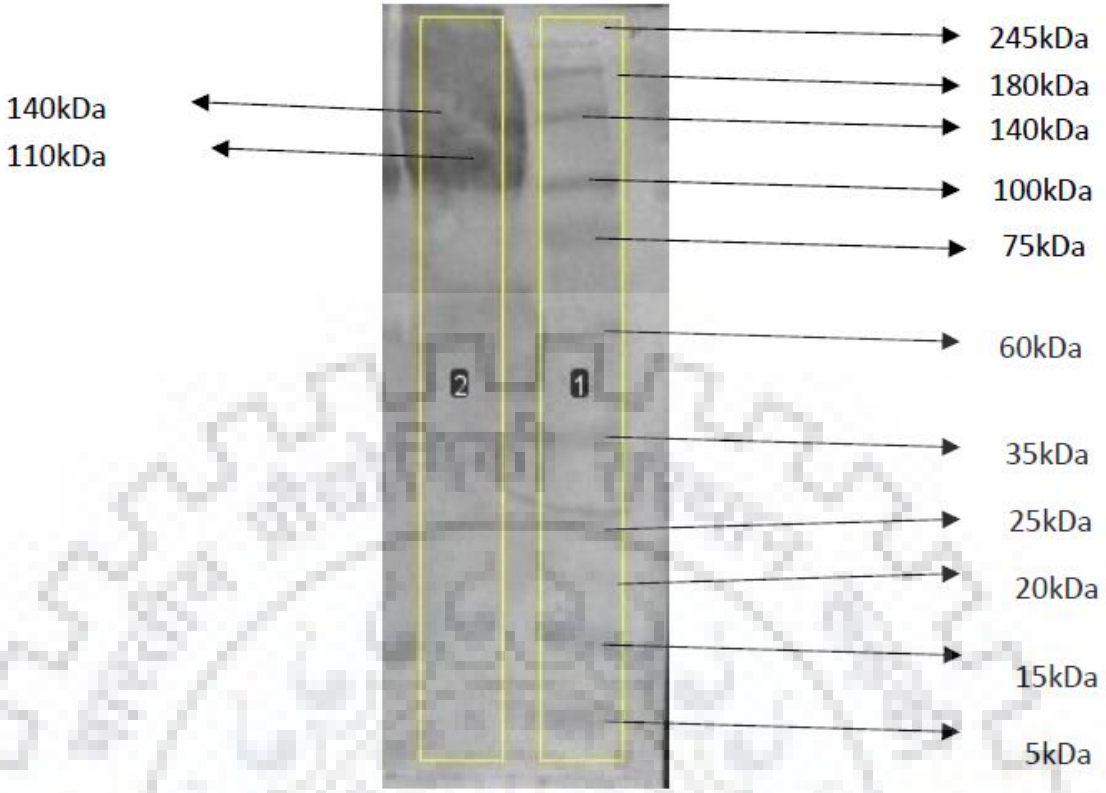


Figure 39. Western blot of HeLa cell Lysate

4.1.16 Checking Masking of Q-dots' signal by Saliva with and without inhibitor

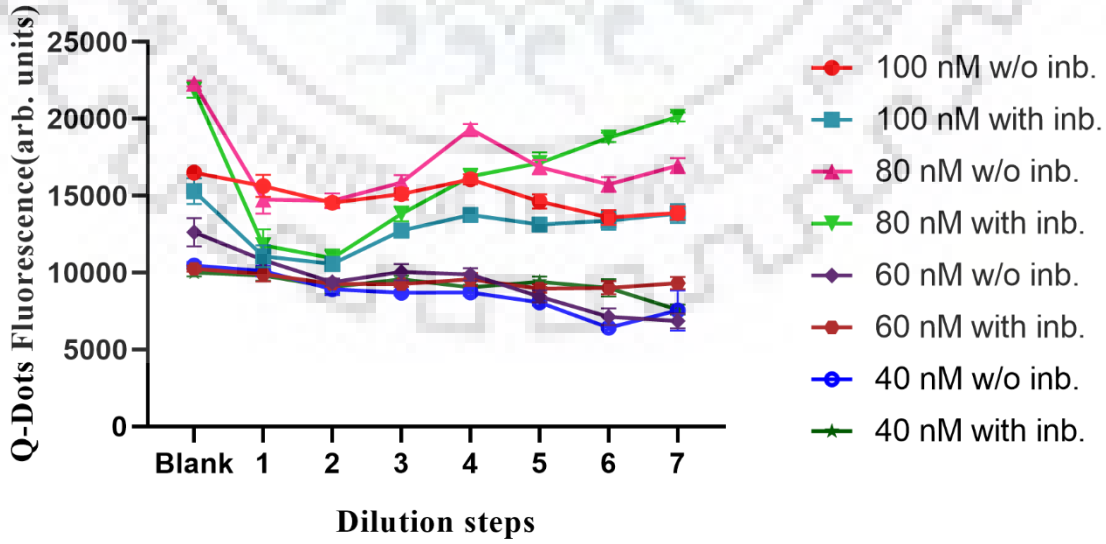


Figure 40. Graph showing change in difference in fluorescence of Q-Dots' spots of after adding solutions of different concentration of Saliva without inhibitor (w/o inb.) and with inhibitor (with inb.

- For most of the samples masking of fluorescence is more by with inhibitor compared to without inhibitor except at lower concentration solutions.

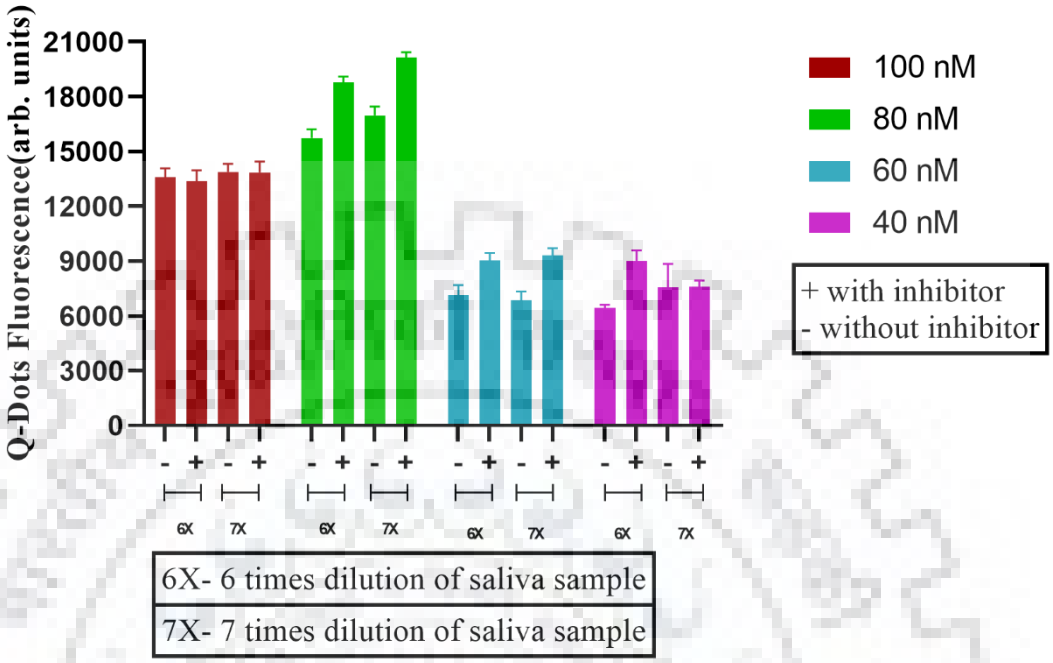


Figure 41. Bar graph showing change in difference in fluorescence of Q-Dots' spots after adding six- and seven-times diluted solutions of Saliva with inhibitor (+) and without inhibitor (-)

- Masking of fluorescence is more by without inhibitor solutions than with ones for 80 nM, 60 nM and 40 nM Q-Dots' at 6^{th} and 7^{th} dilutions.
- The concentration of Saliva is to be lowered more to get a better difference in signal.

4.1.17 Checking stability of spotted Q-dots

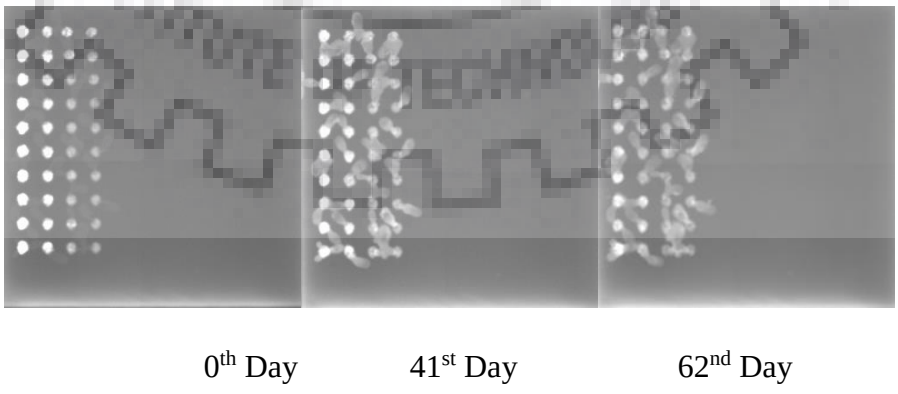


Figure 42. Checking stability of spotted unblocked Q-Dots

- During storage of the spotted Quantum dots over a month swelling of the spots can be seen. This is due to diffusion of the Quantum dots and the solution through the gap of nitrocellulose membrane.
- Maybe the gap in nitrocellulose membrane mesh is slightly bigger than the size of Quantum dots. Complete immobilization of Q-dots is needed.

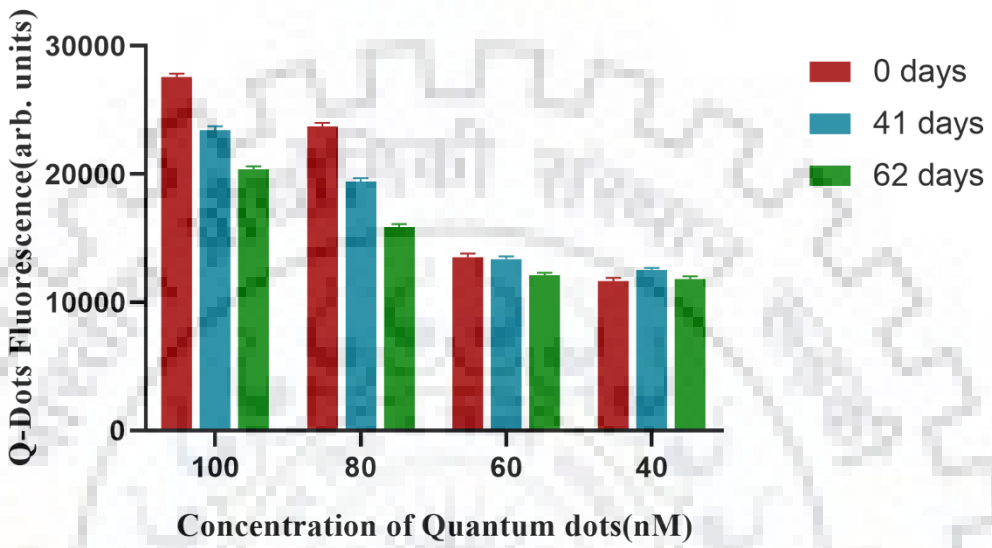
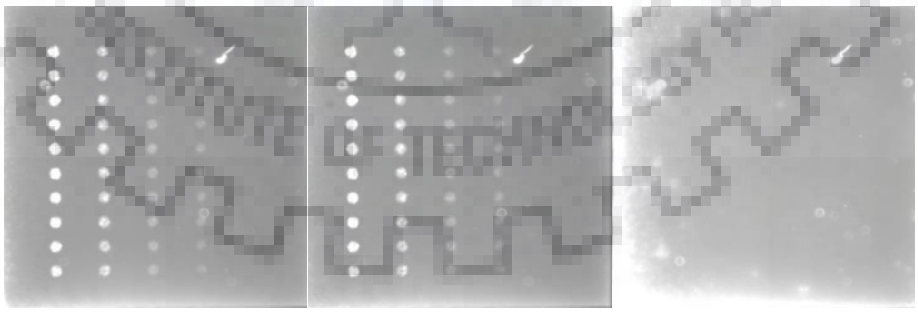


Figure 43. Bar Graph showing change in fluorescence of Q-Dots' spots over a period

- Over 62 days decrease in fluorescence of 100 nM and 80 nM Q-Dots spots is seen. The decrease is not very high.
- For 60 nM and 40 nM there is hardly any decrease in fluorescence.



0^{th} Day 41^{st} Day 62^{nd} Day

Figure 44.Checking stability of spotted blocked Q-Dots

- After adding 3% blocking buffer there is no swelling of the spots till one and half month. There is little decrease in signal for 60 nM and 40 nM spots.
- After two months there is complete loss of fluorescence of all the Quantum dots' spots.

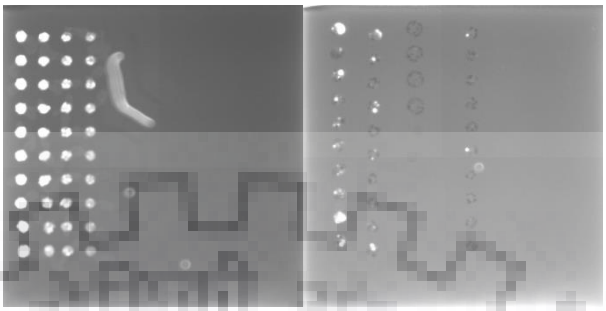


Figure 45. Spotting of 1.5 months and 2.5 months old Q-dots solution

- Spotting of Quantum dots solution which was stored in microwell plate shows Quantum dots have lost their fluorescence in the period. This is mostly due to instability in diluted condition leading to agglomeration of quantum dots.

4.2 Synthesising nanocarriers for inhibitors

4.2.1 Test 1

Sample	Ratio	Hydrodynamic dia.(nm)	Polydisp—ersity Index (5)	Peak Intensity 1 (nm)	Peak Intensity 2 (nm)	Peak Intensity 3 (nm)	Zeta Potential(mV)
Sa	1:1	167.43	24.28	190.16	---	---	-30.62
Sb	2:1	137.059	27.01	158.36	12.17	---	-30.78
Sc	3:1	434.26	10.99	109.95	1281.53	---	-28.85
Sd	4:1	2395.74	21.34	69.170	1086.21	0.2511	-27.97
Se	5:1	4987.64	28.77	63.78	0.326	---	-25.227

Table 32. Diameter of niosomes formed by changing flow rate ratio for test 1

- As the flow rate ratio changes from 1:1 to 5:1 the size of niosomes should decrease.
- In Test 1 the size decreases from 1:1 to 2:1 and then it starts increasing. Also, from 2:1 to 5:1 instead of having only one peak there are 2-3 peaks.
- In order to get uniformity in decrease of niosomes size and to get lesser PdI value concentration of lipid components are doubled in Test 2.

4.2.2 Test-2

Sample	Ratio	Hydrodynamic dia.(nm)	Polydisp—ersity Index(5)	Peak Intensity 1 (nm)	Peak Intensity 2 (nm)	Peak Intensity 3 (nm)	Zeta Potential(mV)
Sa	1:1	153.76	11.01	127.53	---	---	-35.35
Sb	2:1	108.80	27.14	130.83	11.039	---	-23.62
Sc	3:1	66.58	27.71	84.32	958.77	---	-34.96
Sd	4:1	117.56	18.03	120.35	2084.27	---	-25.73
Se	5:1	111.27	17.18	100.17	1167.54	4.800	-26.86

Table 33. Diameter of niosomes formed after changing flow rate ratio for test 2

- In Test 2 the size decreases from 1:1 to 3:1 and then it starts increasing from 4:1, finally decreases for 5:1. Also, from 2:1 to 5:1 instead of having only one peak there are 2-3 peaks.
- In Test 1 and 2 result are good only for 1:1 ratio.
- In Test 2 1:1 niosomes' size and PdI decreased but the zeta Potential is more negative than -30mv.
- Different concentration of Drug is loaded in the niosomes of 1:1 ratio.

4.2.3 Test 3

Sample	Concentration	Hydrodynamic dia.(nm)	Polydisp—ersity Index (5)	Peak Intensity 1 (nm)	Peak Intensity 2 (nm)	Peak Intensity 3 (nm)	Zeta Potential(mV)
Sa	1 mM	3678	33.9	100.2	2087	---	-26.3
Sb	1 µM	50836	53.4	7196	106.9	4447.21	-39
Sc	1 µM	1912.6	23.8	127.50	10409	---	-35.3
Sd	1 nM	19802	27	134.02	---	---	-38.1
Se	1 nM	141690	24.1	5296	88.04	---	-25.1

Table 34. Diameter of niosomes formed under 1:1 flow rate ratio and loaded with drug.

- The size and PdI values of niosomes loaded with drugs are too large. This could be due to instability of the niosomes.

5. Conclusion and scope of future work

5.1 Conclusion

The main aim of the thesis was to explore the capability of Quantum dots to miniaturise microarray assay for Heat shock protein inhibitors and to synthesise niosomes using microfluidic channels as nanocarriers for the inhibitors.

Considering the present work basic principle of microarray is achieved. That is there is a difference in masking of Quantum dots' fluorescence between presence and absence of HSP inhibitor. The technique not only worked for purified protein (*Xanthomonas* htpg) but also for HSP present in HeLa cell lysate and Saliva. This method overcomes problems associated with previous HSP microarray assay. In which the dye like Cy3 attached to ATP used to interfere with attachment of ATP to the protein. Protein microarray slides can't be stored for a longer time. Thus, with further optimization Quantum dots can be used for assays of various natural compounds as inhibitors for various Heat shock proteins.

Microfluidic channels in the nanoassemblr wasn't suitable for making inhibitor loaded niosomes. Also, it is an expensive method because microfluidic channel cassette needs to be changed frequently.

5.2 Scope for future work

Future work involves optimization of the current methodology and slide surface to reduce background noise and get better signal. Storage condition optimization to increase shelf life of spotted slide. More experiments are to be done to reduce statistical errors. Extending the technique to other type of purified Heat shock proteins, blood and other body fluid's HSP.

Using traditional methods to make niosomes that are loaded with HSP inhibitors which are compounds assayed by Quantum dot microarray.

6. References

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