

Colorimetric detection of UCHL1 using gold nanoparticles for rapid diagnosis of brain injury

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The Degree of Master of Technology/ Doctor of Philosophy



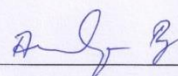
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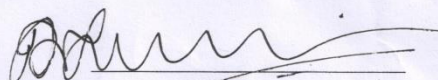
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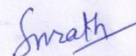
This thesis entitled "Colorimetric detection of UCHL1 using Gold nanoparticles for rapid diagnosis of brain injury" – by – Srishti Agarwal – is approved for the degree of Master of Technology/ Doctor of Philosophy from IIT Hyderabad.



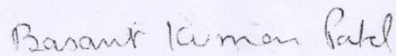
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Declaration

I declare that this written submission represents my ideas in my own words, and where others' ideas or words have been included, I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will be a cause for disciplinary action by the Institute and can also evoke penal action from the sources that have thus not been properly cited, or from whom proper permission has not been taken when needed.



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As my thesis work is finally shaped, I would like to express my feelings to those who helped and supported me in fulfilling my goal.

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Abstract

Ubiquitin C-terminal hydrolase-1 (UCH-L1), an endoprotease that cleaves the specific peptide bond between the ubiquitin molecules, is released in the serum and cerebrospinal fluid (CSF) following severe brain injury. The level of UCHL1 in serum and CSF is inversely proportional to the chance of survival and therefore an important biomarker for brain injury. We developed a sensitive and accurate colorimetric assay using gold nanoparticles (30nm) based on surface plasmon resonance-dependent absorption of visible light at 520nm. Synthesising a specific ubiquitin polypeptide substrate for UCHL1 with two terminal thiol groups, we allowed gold nanoparticles to interact. This resulted blue colour aggregation of substrate polypeptide and nanoparticles with an absorption maxima of 600nm. On addition of UCHL1 to these aggregate, substrate polypeptide molecules was cleaved at the specific position and clustered gold nanoparticles became dispersed resulting the absorption maxima shifting to 520nm and changing the blue colour to red. Presently severity of brain injury is diagnosed using neuro-imaging such as CT scanning and MRI. Simple and inexpensive biochemical assay that we developed might help the physicians to quickly determine severity of the damage, monitor the progress of the treatment and ultimately predict the risk of death of individual patients.

Chapter 1

Introduction and Objectives

1.Introduction:

Ubiquitination and deubiquitination: Ubiquitin is a 76-amino acid polypeptide found in all eukaryotes. The covalent attachment of ubiquitin to the cellular proteins targets them for proteasomal degradation. Ubiquitin-mediated protein modification also plays a crucial role in various cellular processes, including stress response, signal transduction and cell differentiation. By contrast, the deubiquitination reverses the ubiquitination process and is carried out by deubiquitinating enzymes (DUBs). DUBs play two key roles in the ubiquitin pathway. First, ubiquitin is always expressed either as a pro-protein fused to one of two ribosomal proteins or as linear polyubiquitin, consisting of multiple copies of monoubiquitin that must be processed to yield the mature ubiquitin monomer. Second, DUBs reverse the ubiquitination or ubiquitin-like modification of target proteins. Thus far, about 100 human DUBs have been identified, and based on the sequence similarity, they have been classified into ubiquitin C-terminal hydrolases (UCHs) and Ub-specific proteases (UBPs). Both classes are thiol proteases that hydrolyze the isopeptide bond between the substrate and the C-terminal Glycine 76 of ubiquitin.

The UCH family of DUBs is generally small in size (~20-30kDa), and they cleave ubiquitin from relatively small protein substrates of up to 20-30 amino acids. The sequences are well conserved across species with approximately 40% identities and they are structurally similar to the papain cysteine proteases. Like papain family of cysteine proteases, the active site catalytic triad of UCH family of DUBs consists of

Cysteine, Histidine and Aspartate. Structural analysis also revealed a 20 aminoacid residues loop near the active site (called ‘active-site crossover loop’) which plays an important role in substrate binding and induces a conformational rearrangement of the active site in order for hydrolytic activity. Among the human UCHs, UCHL1 is one of the most abundant proteins in the brain and immuno-histological experiments demonstrate that it is localized exclusively to neurons [37]. UCHL1 associates with ubiquitin in neuronal cells and this association is important for the maintenance of mono-ubiquitin levels in neurons. UCHL1 effectively up-regulates ubiquitin levels at the post-translational level, and this upregulation is probably based on the inhibition of ubiquitin degradation by UCHL1 [28].

1.2. Brain damage biomarker: Severe head injury that damages internal brain tissue is one of the leading causes of death and disability. Unlike other organ-based injuries or diseases where rapid diagnosis is available, no diagnostic tests have been developed for serious brain injury. Currently, diagnosis depends on a variety of measures including neurological examination and neuroimaging such as CT scanning and MRI, all of which have some limitations. CT scanning has low sensitivity to diffuse brain damage and the MRI is not widely available and rather expensive. A blood biomarker-based simple and inexpensive diagnosis will help the physician to quickly determine severity of the damage, monitor the progress of the treatment and ultimately predict the chance of survival of patients. Previous studies established few protein biomarkers for brain injury [3, 9]. These include neuron-specific enolase (NSE), glial protein S-100 β , myelin basic protein (MBP) and a deubiquitinase, known as ubiquitin carboxy-terminal hydrolase-1 (UCHL1) [10, 26, 31, 39]. Although NSE, S100 β , and MBP was shown to be released in the blood following brain damage, conflicting results made their utility uncertain [20, 25]. On

the contrary, UCHL1 protein is present exclusively in the neuron (unlike NSE and S100B, which are also present in non-neural tissues) [10]. UCHL1 (historically known as PGP 9.5) was first detected as a 'brain-specific protein' over 30 years ago [15]. It has been shown that UCHL1 is released in the cerebrospinal fluid (CSF) after ischemic and traumatic brain injury in rats [23]. Clinical study with several brain injury patients established that higher level of UCHL1 in serum and CSF is inversely proportional to chance of survival [4, 27, 30]. Since there are no other endopeptidases known to be present in the serum and CSF, a robust assay to detect UCHL1 in brain injury patients would help in monitoring the progress of the treatment.

1.3. UCH-L1 as Brain damage biomarker: The Ubiquitin C-terminal hydrolases (UCH) are a group of DUBs that specifically hydrolyse the peptide bond following the C-terminal Gly of Ubiquitin. To date, four UCH enzymes have been identified from human cells: UCH-L1, UCH-L3, UCH-L5 and BAP1 (BRCA1-associated protein 1). Among these, UCH-L1 is a highly specific neuronal protein that is concentrated in the grey matter region of the brain [5]. This 24kDa enzyme constitutes 10% of neuronal cytosolic proteins [15]. Physiological role of UCH-L1 is to remove conjugated ubiquitin from degradation-targeted proteins which is important for protein turnover in neurons. Often, misfolded proteins, if not appropriately catabolized, could result in protein aggregation which is neurotoxic. Human studies have also linked UCH-L1 to degenerative CNS diseases such as Parkinson's disease and Alzheimer's disease [11]. UCH-L1 has also been shown to be biomarker of neuronal loss during brain haemorrhage [21]. Presence of UCH-L1 in the CSF after serious brain injury has been discovered recently and clinical studies established the strong correlation between concentration of UCHL1 and

outcome of the treatment [4, 27, 30]. UCH-L1 is an excellent biomarker for several reasons: (1) UCH-L1 is highly expressed almost exclusively in the brain. (2) UCH-L1 can cross brain-blood barrier as it is a small protein with a molecular weight of about 24 kDa and has a compact and almost globular shape [16]. (3) It is stable in the biofluid as western blots of CSF fluids revealed that it remains as an intact protein with no detectable breakdown product [4]. UCH-L1 level could increase 25-50 fold following injury and detectable within 6 hrs of injury and peaks within the first 24 hrs [30]. Ubiquitination and deubiquitination play pivotal roles in many biological processes. Although UCHL1 could be an important biomarker, till date there is no simple method to detect UCH-L1 during brain injury.

1.4. Gold nanoparticle-based biomarker detection. Optical properties of gold NPs can be used as a means to obtain information on processes occurring in the solution. It is well established that gold NPs of ~10-40nm diameter display an absorption at 520nm due to the excitation of the surface plasmon which shifts to longer wavelengths (600nm) upon cluster formation. The progress of the aggregate formation can be followed with the naked eye as the colour of the solution changes from red to blue. The possibility to control nanoparticle aggregation has been exploited in many areas of chemical sensing and enzyme bioassays. For example, gold NPs have been used for assaying superoxide dismutase, phosphatase, Acetylcholine esterase, lactamase, protease, lysozyme, nuclease, thrombin and glucose oxidase [6-8, 13, 14, 18, 24, 29, 35, 36, 38]. However, gold NPs have not been used to assay any ubiquitinating or deubiquitinating enzyme. Thus, developing gold NP based UCH-L1 assay method for detection in clinical samples is the main aspect of the proposed project.

In India, active research is being carried out on gold NPs based chemical sensing [29]. However, there has been no study anywhere in the World on utilizing deubiquitinating enzymes for clinical application. This gives a broad room to apply the existing knowledge of gold NPs and biochemistry of UCH-L1 as biomarker for brain injury. Therefore, research initiative in this direction in India will be useful as a new area in translational research.

1.5. Rationale of gold nanoparticle based biomarker detection. In this proposal we will exploit two unique properties of the gold nanoparticles (gold NPs): **(1)** It is well known that the gold NPs of 10-50 nm diameter exhibit localized surface plasmon resonance (SPR) that results in absorption at 520 nm displaying intense red colour. On aggregation of the gold NPs, the optical properties change and absorption maxima shift to longer wavelengths of 600nm turning the colour to blue. Such clearly distinguishable and predictable colour change during gold NP aggregation or re-dispersion provides would provide an elegant platform for developing the absorption-based colorimetric detection system that can be performed without any sophisticated instruments. Because of the extremely high extinction coefficients of gold NPs, this assay would provide sensitivity comparable to conventional analytical methods such as ELISA. **(2)** It was shown that gold NPs also interact strongly with thiol-groups and peptide featuring a head and tail thiol-groups causes aggregation of gold NPs [12]. These two characteristics of the gold NPs can be utilized in an artificially designed polypeptide substrate containing two thiol-groups at the two termini for UCHL1 endoprotease detection.

2. Objective

We hypothesize that presence of the UCHL1 substrate peptides will cause thiol-mediated interaction and aggregation of gold NPs resulting in blue colour. On addition of sample containing UCHL1 these substrate peptide molecules will be cleaved at the specific position by endopeptidase activity of UCHL1 and gold NPs will become dispersed resulting the changing of colour to red. A pictorial overview of the proposed assay is depicted. The proposed project aims to develop a rapid and simple assay using gold nanoparticle for the detection of presence of UCHL1 in the clinical samples. In order to achieve this objective, the project will be focused on the following specific objectives:

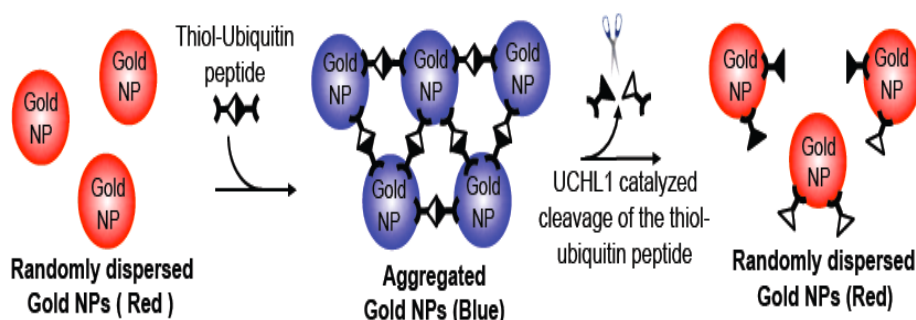


Figure 1. Schematic representation showing the strategy of proposed UCHL1 assay using Gold NPs. Addition of thiol-ubiquitin peptide induces aggregation of Gold NPs (detected by blue colour) and UCHL1 mediated endoproteolytic cleavage of the peptide results dispersion of the Gold NPs (detected by red colour).

Objective 1: Gold nanoparticle-based assay for detection of UCH-L1

Specific aim-1: Synthesis of a specially designed thiol-ubiquitin substrate specific to UCHL1: The polypeptide substrate used for detecting the UCHL1 activity will be modified to incorporate two terminal thiol groups which will be contributed by cysteine residues. Site-directed mutagenesis approach will be used to incorporate these two cysteine residues.

Specific aim-2: Aggregation of Gold NP by using the thiol-ubiquitin substrate: The appropriate concentration of thiol-containing peptide substrate required and optimal buffer composition that induces aggregation of gold NPs without causing precipitation by nonspecific aggregation will be determined empirically. The thiol-ubiquitin substrate-induced aggregation of gold NPs process will be monitored by recording the change of absorbance as absorption maxima (530nm) of dispersed gold NP colloids is different from aggregated gold NPs (650nm).

Specific aim-3: Cleavage of thiol-ubiquitin substrate by UCHL1 and induced dispersion of Gold NP: Thiol-ubiquitin substrate-induced gold NPs aggregate will be used as substrate for UCHL1 mediated cleavage. The decrease of absorbance at 650nm and increase at 530nm and colour change from blue to red would be the indicator of UCHL1 mediated dispersion of Gold NPs.

Chapter 2

Materials and Methods

Buffers, Media and Solutions:

Deionised water was used for all buffers solutions and media

LB medium

1 % w/v Tryptone (HIMEDIA,#G010)

0.5 % w/v yeast extract (HIMEDIA,# RM027)

SOC medium

2 % w/v Tryptone (HIMEDIA,#G010)

0.5 % w/v yeast extract (HIMEDIA,# RM027)

TB medium

1% w/v yeast extract(HIMEDIA,# RM027)

0.5% w/v tryptone(HIMEDIA,#G010)

TE (Tris-EDTA) [50X]

1 mM Tris-HCl pH 8.0

0.1 mM EDTA pH 8.0

A 10 x stock solution was routinely used to prepare 1 x TE.

Isopropyl β -D-1-thiogalactopyranoside [1M]

IPTG (Sigma,# I6758-1G)-4.7g

H₂O-20ml

SDS [20%]

SDS: 20g; dissolved in 100 ml water.

Ammonium Per Sulphate [20%]

APS- 20g

H₂O- 100ml

Resolving gel buffer:

1.5 M Tris-HCl, pH 8.8

Tris (Sigma) 12.1g

HCl 3-4ml

Makeup vol to 100ml

Stacking gel buffer:

0.5 M Tris-HCl (for stacking gel), pH 6.5

Tris(sigma) 12.1g

HCl 6-7ml

Makeup vol to 100ml

30% Acrylamide:

Acrylamide: 29 %

bis-Acrylamide: 1 %

10X SDS-PAGE gel running buffer:

Tris base 30.3 g

Glycine 144 g

SDS 10 g

H₂O 1000 ml

3X SDS-PAGE loading dye:

1M Tris-HCl, pH 6.5 - 2.4 ml

20% SDS -3 ml

Glycerol (100%) -3 ml

B-mercaptoethanol -1.6 ml

Bromophenol blue -0.006g

H₂O -10 ml

SDS-PAGE staining solution:

Coomassie blue R250- 0.4 % w/v

Methanol- 30 % v/v

Acetic acid 10 %v/v

SDS-PAGE destaining solution:

Methanol- 30 % v/v

Acetic acid 10 %v/v

Total Protein extraction buffer:

50 mM Tris (pH 8.8)

250 mM NaCl

0.05% Triton-X

UCHL1 10X Assay Buffer:

Tris-HCl (pH-8.0) -200mM

EDTA- 5mM

DTT - 50Mm

Glycerol- 40-50%

Bacterial strains: DH5 α was the recipient strain for the plasmids used in cloning. BL21(DE3) pLysS was the strain used for recombinant protein expression.

General Methods

Preparation of competent *E. coli* cells by calcium chloride method: Competent *E. coli* cells of DH5 α and BL21 (DE3) pLysS, were prepared as described by Alexander [1] using MnCl₂ and CaCl₂. A single colony of *E. coli* strain was inoculated in 25 ml of sterile 2x LB medium (in 250ml Erlenmeyer flask) and incubated in shaker at 200rpm/30°C overnight. 1 ml of this preinoculum was added to sterile 200 ml 2x LB medium and grown in incubator shaker at 30°C/200rpm/4 hrs. The culture was then chilled for 1hr at 4°C and cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The supernatant was discarded and the cell pellet was resuspended in 12 ml of acid salt buffer-A (ASB-A, Na.acetate-40 mM, CaCl₂-100 mM, MnCl₂-70 mM, pH 5.5) and incubated on ice for 1 hr. The ASB treated cells were then pelleted by centrifugation carried out at 3500 rpm for 15 min at 4°C and resuspended in 4ml of ASB-B (Na.acetate-40 mM, CaCl₂-100 mM, MnCl₂-70 mM, 15% glycerol, pH 5.5) and were stored (aliquots of 100 μ l) at -86 °C for future use.

Transformation of competent *E. coli* cells: DH5 α or BL21 (DE3) pLysS competent *E. coli* cells were prepared as before. 10 ng of pure plasmid DNA or 5 μ l of a ligation mix were added to 50 μ l aliquots of cells and the mixture was incubated on ice for 30 minutes. After a 42°C heat shock for 30 seconds, the

cells were briefly incubated on ice for 5 minutes before 200 μ l of SOC media was added. This cell suspension was then incubated at 37°C for 1 hour, shaking at 250 rpm. After this incubation, 20 μ l and 200 μ l aliquots were plated on LB plates containing 100 μ g/ml ampicillin and the plates were incubated at 37°C overnight.

Quick transformation of BL21 (DE3) pLysS competent cells: BL21 (DE3) pLysS competent *E.coli* cells were prepared as before. 5 μ l of a plasmid DNA was added to 50 μ l aliquots of cells and the mixture was incubated on ice for 5 minutes. After a 42°C heat shock for 30 seconds, the cells were briefly incubated on ice for 5 minutes. After this incubation, entire transformation mix was plated on LB plates containing 100 μ g/ml ampicillin and the plates were incubated at 37°C overnight.

Plasmid mini-prep: Mini-preps of plasmids were performed using the Plasmid Mini-Prep kits (Thermo Scientific #K0503) according to the instructions of the manufacturer. Standard culture volumes were 2 ml for minipreps. The purified plasmid DNA was analyzed by restriction digestion and agarose gel electrophoresis to confirm the identity of the obtained plasmid. Miniprep of plasmid DNA was also carried out by alkaline lysis method[32].

Restriction digests and ligation reactions: All restriction enzymes were purchased from New England Biolabs and used according to the manufacturer's instructions. Ligation reactions were performed using T4-DNA ligase (New England Biolabs, cat #M0202S), used as recommended by the manufacturer. Whole ligation reactions were used for transformation into *E. coli*(DH5 α).

Agarose gel electrophoresis: Horizontal agarose gel electrophoresis unit (BIO-RAD #166-4000EDU) was used for analysis of DNA. All agarose gels were 0.8%

w/v agarose (SeaKem LE, LONZA #50005) in 1xTAE (40mM Tris-acetate; 1mM EDTA pH 8.0). The samples were loaded in 1x loading dye (6x stock: 0.25% bromophenol blue; 0.25 xylene cyanol FF; 30% v/v glycerol). Gels also contained 1µg/ml ethidium bromide to allow visualization of the DNA under UV light. The gels were viewed under Gel Documentation System (Syngene). Gels were run at 100V. DNA ladder of 1 kb (Thermo Scientific, # SM0311) was used for fragment size determination.

Purification of DNA from agarose gels (gel extraction): DNA was purified from agarose gels using GeneJET gel extraction kit from Fermentas Life Sciences cat #K0692, as instructed by the manufacturer.

SDS-PAGE: Expression of recombinant protein was checked in 12.5% SDS-PAGE according to the standard protocol. SDS-PAGE was carried using discontinuous buffer system as described by Laemmli using slab gel electrophoresis apparatus [17]. Throughout this study 1.5 mm thick 12% polyacrylamide (30:0.8, acrylamide to bisacrylamide ratio) gel containing 0.1% SDS was used for electrophoretic separation of proteins. The protein samples were mixed with an equal volume of 3X loading dye (2.4 ml 1M Tris-HCl pH 6.5, 3 ml 20% SDS, 3 ml 100% Glycerol, 1.6 ml β-mercaptoethanol, 0.006 g Bromophenol blue) and heated at 100 °C for 10 min and were loaded into the wells of precast polyacrylamide gel. Electrophoresis was performed at a constant voltage of 100 or 120V using 0.025M Tris and 0.2 M glycine buffer pH 8.8 containing 0.1% SDS. After the electrophoresis, the gel was stained with coomassie brilliant blue (0.4% w/v Coomassie blue R250, 30% v/v Methanol, 10 % Acetic acid)

Checking the expression of the recombinant clones: *E.coli* BL21(DE3) cells transformed with recombinant plasmids were grown and induced for protein

expression as described by Studier *et al* [33, 34]. A single colony was inoculated in 6 ml of Luria-Bertani medium containing 100µg/ml ampicillin and grown for 4 hrs at 37°C in a screw-cap inoculation vial. From this culture 2ml culture was recovered as a reference for uninduced *E.coli* extract and the remaining 4ml culture was induced with 0.1mM (final concentration) isopropyl-β-D-thiogalactopyranoside (IPTG). Following induction the culture was further grown for 4 h at 37°C and 1.5ml culture was recovered from the induced sample as a reference for induced *E.coli* extract and 750 µl was recovered from uninduced culture. The induced and uninduced cells were harvested and resuspended in 100µl protein extraction buffer (50 mM Tris pH 8.8, 250 mM NaCl, 0.05% Triton-X) and lysed by sonication. From the sonicated samples, 60µl were added to 30µl of 3x sample loading dye [17], boiled for 10 min and were used for SDS-PAGE analysis in 12% gel. The electrophoresis was carried out at 100 volts and the proteins were stained with Commassie brilliant blue.

Cloning of human *UCHL1*

Polymerase Chain Reaction (PCR) for UCH-L1 gene amplification: PCR master mix was prepared by adding water, buffer, dNTPs, primers and Taq DNA polymerase and template DNA (10 µl).

- 10 µl of 5 x Phusion High Fidelity Buffer
- 1 µl of dNTPs (10 mM each)
- 1µl of primer-UCHL1-BamHI-Sen and UCHL1-SalI-HindIII-Anti (100 µM)
- 1 µl of Phusion High Fidelity DNA Polymerase
- 1µl of template DNA(pEGFP1-hUCHL1)

PCR grade water was used to reach the final reaction volume 50µl. PCR temperature program for UCHL1 gene amplification:

1. 98°C, 30 sec
2. 98°C, 10 sec
3. 57°C, 30 sec
4. 72°C, 1 min
5. 72°C, 10Min
6. 4°C, Hold; Step 2-4 were repeated for 32 cycles

Ligation and screening: Full length UCH-L1 was PCR amplified in 50 µl reaction volume by using Phusion high fidelity DNA polymerase (New England Biolabs, # M0503S), 1 µl of pEGFP.C3.hUCHL1 as template and specific primers (UCHL1-BamHI-Sen and UCHL1-SalI-HindIII-Anti). The amplification was examined by 0.8% agarose gel electrophoresis using 1µg/ml ethidium bromide to allow visualization of the DNA under UV light. DNA ladder of 1kb (Thermo Scientific, # SM0311) was used for fragment size determination. The full length *UCHL1* product and plasmid pRSETA were sequentially digested by *BamHI* and *HindIII* at 37°C/4h, in 40µl reaction volume containing each 1µl of restriction enzyme. The entire digestion mixture was loaded in 0.8% agarose gel. After electrophoresis the appropriate size bands were visualized on low intensity UV (340nm) light of UV trans-illuminator and excised using a sterile scalpel. DNA was eluted from the agarose gel piece according GeneJET kit protocol (Fermentas Life Sciences, cat #K0692). Ligation was set-up in 10 µl reaction with 7.5 µl of restriction digested and gel eluted PCR DNA(11.65 ng/µl), 0.5 µl of restriction digested and gel eluted vector(28.45 ng/µl), 1µl of ligation buffer containing ATP and 1 µl of T4-DNA ligase (New England Biolabs, cat #M0202S) and incubated at 16°C/overnight. The entire ligation mixture was transformed to 50 µl of DH5α competent cells and grown on LB-plates containing 100 µg/ml ampicillin. After overnight incubation at 37°C, 9 colonies were selected and grown in 2ml LB-ampicillin. Further, plasmid was

purified by alkaline lysis method [38]. To confirm the presence of UCH-L1 and also orientation , restriction analysis of the slow moving clones was performed using enzymes *NcoI* and *ScaI*.

Table 1

Purpose	Name	Sequence (5' – 3')
Forward primer for cloning UCHL1	UCHL1-BamHI-Sen (40nt)	CCTGGGATCCATGCAGCTCAAGCCG ATGGAGATCAACCCC
Reverse primer for cloning UCHL1	UCHL1-SalI-HindIII-Anti (43nt)	AAAAGTCGACAAGCTTTTAGGCTGC CTTGCAGAGAGCCACGGC
Forward primer for cloning Ubiquitin	Ub-NdeI-Sen (38nt)	GATATACATATGCAGATCTTCGTGA AGACTCTGACTGG
Reverse primer for cloning Ubiquitin	Ub-HindIII-Anti (56nt)	GCCTAGAAGCTTCTAGCAATGATGA TGATGATGATGCTTCCCACCTCTGA GACGGAGTACC
Forward primer for Ub-K63C	Ub-K63C-Sen (52nt)	CCCTGTCTGACTACAACATCCAGTG TGAGTCCACCTTGCACCTGGTACTC CG
Reverse primer for Ub-K63C	Ub-K63C-Anti (52nt)	CGGAGTACCAGGTGCAAGGTGGACT CACACTGGATGTTGTAGTCAGACAG GG

Purification of UCH-L1

The recombinant soluble protein was purified by Ni-NTA affinity chromatography. For this, few BL21+pRSETA-UCHL1 transformed colonies were inoculated into 100ml LB-broth with 100 µg/ml ampicillin and grown on incubatory shaker at 37°C at 200 rpm. The grown culture was 20 fold diluted into 2L fresh TB-broth with 100 µg/ml ampicillin. Incubation was carried out at 37°C at 200 rpm for 3 hrs. The culture was then induced with 1mM IPTG and kept for 5 hrs at 30°C at 200 rpm. Cells were harvested after centrifugation at 8000 rpm for 15 minutes at 4°C. The cell pellet was resuspended in 20ml of 50mM Tris-HCl buffer (pH 8.0) containing 300mM sodium chloride, 0.1%

Triton-X, 1mM Imidazole and sonicated for 30 minutes. The sample was then centrifuged at 14,000 rpm for 20 minutes at 4°C to remove insoluble debris. The soluble fraction was mixed with 2ml of Ni-NTA resin pre-equilibrated with the same buffer and allowed to rotate in rotary shaker for 4h to allow the protein to bind to the resin. The sample was then centrifuged at 1000 g for 5 minutes at 4°C and the washing is carried out in 3 steps with 75 ml wash buffer (50 mM Tris,pH8.0, 350 mM NaCl, 0.1% Triton-X and 5 mM Imidazole). Centrifugation was carried out at 1000g for 4 minutes at 4°C. Bound protein was eluted with elution buffer (10 mM Tris,pH8.0, 100 mM NaCl, 400 mM Imidazole). 1.5ml of elute1, 1ml of elute 2 and 1ml of elute 3 was collected. All elutes were checked in 12% SDS-PAGE.

Cloning of Ubiquitin with C-terminal extension (Ub)

Deubiquitinase activity of UCHL1 was checked *in vitro* by using the Ub having a C-terminal extension peptide (Lys-His-His-His-His-His-His-Cys). UCHL1 is reported to cleave the bond present between C-terminal glycine of Ubiquitin and a short peptide, preferably starting with lysine. Therefore, the peptide substrate was designed with lysine after the last amino acid of Ub (Glycine) followed by six histidine and a cysteine. This Ub C-terminal extension peptide would help in Ni-NTA purification of ubiquitin, detection using anti-His tag antibody and analysis of endopeptidase activity of UCHL1.

Polymerase Chain Reaction (PCR) for Ub gene amplification: PCR reaction of 25µl for amplification of Ub contained

- 5 µl of 5 x Phusion High Fidelity Buffer
- 0.5 µl of dNTPs (10 mM each)

- 0.25µl of primer-Ub-NdeI-Sen and Ub-HindIII-Anti (100 µM)
- 0.5 µl of Phusion High Fidelity DNA Polymerase
- 0.5µl of template DNA(pGEX-Ubiquitin)

PCR grade water was used to reach the final reaction volume 25µl. PCR temperature program for Ubiquitin gene amplification:

1. 98°C, 30 sec
2. 98°C, 10 sec
3. 56°C, 30 sec
4. 72°C, 1 min 30 sec;
5. 72°C, 10Min
6. 4°C, Hold; Step 2-4 were repeated for 32 cycles

Ligation and screening: Full length Ubiquitin was PCR amplified in 50 µl reaction volume by using Phusion high fidelity DNA polymerase (New England Biolabs, #M0503S), 1 µl of pGEX- Ubq as template and primers Ub-*NdeI*-Sen and Ub-*HindIII*-Anti. The amplification was examined by 0.8% agarose gel electrophoresis using 1µg/ml ethidium bromide to allow visualization of the DNA under UV light. DNA ladder of 1kb (Thermo Scientific, # SM0311) was used for fragment size determination. The full length Ubiquitin product and plasmid pRSETA were sequentially digested by *NdeI* and *HindIII* at 37°C/4h, in 40µl reaction volume containing each 0.5µl of restriction enzyme. The entire digestion mixture was loaded in 0.8% agarose gel. After electrophoresis the appropriate size bands were visualized on low intensity UV (340nm) light of UV trans-illuminator and excised using a sterile scalpel. DNA was eluted from the agarose gel piece according GeneJET kit protocol (Fermentas Life Sciences, cat #K0692). Ligation was set-up in 10 µl reaction with 6.5 µl restriction digested and gel eluted PCR DNA (8.3ng/µl), 1µl ligation buffer, 1.5 µl restriction digested and gel eluted vector(23.95ng/µl) and 1 µl of T4-DNA ligase containing ATP (New England Biolabs,cat #M0202S) and incubated at 16°C/overnight. The entire ligation mixture was transformed to 50 µl of

DH5 α competent cells and grown on LB-plates containing 100 $\mu\text{g/ml}$ ampicillin. After overnight incubation at 37°C, 9 colonies were selected and grown in 2ml LB-ampicillin. Further, plasmid was purified by alkaline lysis method [32]. To confirm the presence of Ubiquitin in right orientation, restriction analysis of the slow moving clone was performed using enzymes *NdeI* and *HindIII*.

Site directed mutagenesis of Ub at position Lys63 to Cys63 to generate ubiquitin containing two-thiol groups (Ub-SH)

The entire plasmid-containing mutant Ubiquitin was amplified by the two overlapping oligonucleotides that carried the specific mutation. A reaction of 50 μl contained for mutation PCR contained:

- 10 μl of 5 x Phusion High Fidelity Buffer
- 1 μl of dNTPs (10 mM each)
- 0.25 μl of each primer- Ub-K63C-Sen and Ub-K63C-Anti100 μM
- 1 μl of Phusion Q5 hot start DNA Polymerase
- 1 μl of template DNA(pRSETA-Ubiquitin)

PCR grade water was used to reach the final reaction volume 50 μl . PCR temperature program for mutagenesis:

1. 98°C, 30 sec
2. 98°C, 10 sec
3. 56°C, 30 sec
4. 72°C, 1 min
5. 72°C, 10Min
6. 4°C, Hold; Step 2-4 were repeated for 20 cycles

To remove the non-mutated template plasmid, the entire PCR mixture was treated with 1 μl of *DpnI* digestion at 37°C for overnight incubation, which degrades specifically methylated DNA that is template plasmid purified from bacterial host. *DpnI* digested PCR mixture was then transformed to 50 μl DH5 α competent cells and grown on LB agar plates containing 100 $\mu\text{g/ml}$ ampicillin. After overnight incubation the colonies were inoculated to 2ml of LB-ampicillin and plasmid was

purified from grown culture using plasmid purification kit (Fermentas Life Sciences) according to manufacturer's instructions

Purification of Ub and Ub-SH

Purification of Ub: Purification was carried out in one step by affinity purification using Ni-NTA chromatography. Few BL21+pRSETA-Ub transformed colonies were inoculated into 100 ml LB-broth with 100 µg/ml ampicillin and grown on incubatory shaker at 37°C at 200 rpm. The grown culture was 20-fold diluted into fresh 2L TB-broth with 100 µg/ml ampicillin. Incubation was carried out at 37°C at 200 rpm for 3 hrs. The culture was then induced with 1mM IPTG for 5 hrs at 30°C at 200 rpm. Cells were harvested after centrifugation at 8000 rpm for 15 minutes at 4°C. The cell pellet was resuspended in 50ml of extraction buffer (50mM Tris-HCl buffer (pH 8.0), 300mM sodium chloride, 0.1% Triton-X, 1mM Imidazole) and sonicated for 40 minutes. The sample was then centrifuged at 14,000 rpm for 20 minutes at 4°C to remove insoluble debris. The soluble fraction was then heated for 10 min at 70 °C and again centrifuged at 14000rpm/20min/4°C. After this the soluble fraction was mixed with 2ml of Ni-NTA resin pre-equilibrated with the same buffer and allowed to rotate in rotary shaker for 4h to allow the protein to bind to the resin. The sample was then centrifuged at 1000 g for 5 minutes at 4°C and flow through was collected. Washing was then carried out in 3 steps with 75 ml wash buffer (50 mM Tris,pH8.0, 350 mM NaCl, 0.1% Triton-X and 5 mM Imidazole) with 10 min washing on rotor. Centrifugation was carried out at 1000g for 4 minutes at 4°C. Bound protein was eluted with 8ml elution buffer (10 mM Tris,pH8.0, 100 mM NaCl, 250 mM Imidazole). 1.5ml of elute 1,1 ml of

elute 2 and 1 ml of elute 3 was collected. All elutes were checked in 15% SDS-PAGE.

Purification of Ub-SH: The mutant protein containing two thiol groups was purified exactly like Ub described before.

Qualitative analysis of enzymatic hydrolysis of Ub-SH

The *in vitro* assay of UCHL1 activity on Ub substrate was performed by using 48 μ g (15.0 μ M) of UCHL1 and 50 μ g (100 μ M) of Ubiquitin in a 50 μ l reaction in 10X assay buffer. Reaction mix was incubated 2h at 30°C for the activity of UCHL1 to occur. Identical reaction was also carried out with catalytic mutant of C90A UCHL1. Activity of wild type UCHL1 and the mutant UCHL1 on the Ub C-terminal extension peptide was analyzed by Gradient SDS PAGE gel (4-15%) from BIORAD.

Preparation of gold nanoparticles

The synthesis is based on the reduction of gold chloride by sodium citrate at specific concentrations and at a temperature high enough for the sodium citrate to act as a reducing agent (around 80-100°C), and thus causing the gold to be reduced from Au(III) to Au(0). Citrate serves a dual purpose in the synthesis; it serves to both reduce the gold and stabilize the particles. In the zero oxidation state, gold atoms start to nucleate and grow into particles. Usually a stabilizing agent is also added to prevent the particles from aggregating. Because thiol groups bind to gold surfaces with high affinity, most frequently thiol modified ligands are used as stabilizing agents which bind to the surface of the AuNPs by formation of Au-sulfur bonds.

Synthesis of AuNPs with various sizes and shapes can be achieved through judicious choice of experimental conditions and reagents.

Reagents required for synthesis of gold nano-particles of different sizes were: (A) 0.25 mM HAuCl₄ (4.9mg HAuCl₄ (MW 393.83) dissolved in 50ml deionised water). (B) 1% Sodium citrate (0.2g Sodium citrate was added to 20 ml deionized water). 50ml aqueous solution of HAuCl₄ (0.25 mM) was heated to boiling on a stir/hot plate while stirring with the magnetic stir bar. After the solution started to boil, 1.6ml, 0.875ml, 0.625ml, 0.5ml of trisodium citrate (1%) was added to get 10nm, 20nm, 32nm and 41nm AuNP respectively (Table 2). In about 25 s, the boiling solution turned faintly blue (nucleation). After approximately 70 s, the blue color suddenly changed into a brilliant red, indicating the formation of gold particles. The solution was continued to boil and stirred until deep red color (about 10 min) was obtained. While the solution was boiling, a few drops of distilled water was added to keep the total solution volume near 50 mL. The particles formed by this method was allowed to stand for 24h before their use for further studies. Gold nanoparticle solution is stable for months if stored in covered glass containers at room temperature. All concentration determinations were done with UV-Vis measurements.

Table 2.

Volume of HAuCl ₄	HAuCl ₄ (mM)	Vol of 1% sodium citrate	Sodium citrate (mM)	λ max		Average diameter (nm)	
				Reported	Observed	Reported	Observed
50ml	0.25	1.6 ml	1.09	519	522.50	10	10
50ml	0.25	0.875ml	0.59	526	528.50	20	20
50ml	0.25	0.625ml	0.43	529	528.00	32	32

50ml	0.25	0.500ml	0.34	532	532.50	41	41
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Characterization of gold nanoparticles

Electron microscopy: A 2 μL drop of nanoparticle solution at a concentration of 0.1 mg/mL in milliQ H_2O was dried onto a carbon coated grid and viewed under a Leo 912 AB TEM at 120kV, at 40,000 \times magnification.

Aggregation of gold nanoparticles by ubiquitin containing two-thiol groups detected by Surface plasmon resonance

A strong broad absorption band is observed in the absorption spectra of many metallic nanoparticles, especially gold and silver. This occurs because of the coupling of incident electromagnetic radiation into a surface Plasmon, (described as a collective oscillation of the conduction electrons) at the interface between the particle and the medium surrounding the particle. The resulting energy loss is manifested as an absorbance known as surface Plasmon band. Being confined to the surface, this collective oscillation of the free electrons is sensitive to changes in the size of particle and as the diameter gets smaller the energy required to collectively excite motion of the surface Plasmon electrons increases. Gold nanoparticles with diameters greater than 5nm have maxima at longer wavelengths and by varying the particle size one can tune the maximum of the SPR absorbance to be anywhere from 520nm to 1000nm. Any species adsorbed to the nanoparticle surface will manifest a colour change (shift in SPR peak position) proportional to magnitude of the change in refractive index near the nanoparticle surface(40). We carried out surface

modification of 30nm gold nanoparticles with purified Ub-SH peptide. To achieve this, the reaction mix was prepared as follows:

Table 3.

Chemical	Reaction Vol	Concentration
AuNP	200 μ l	50.0 μ M
Ub-SH	15 μ l	0.25 μ M
Water	785 μ l	-
Total =	1000 μ l	-

Ubiquitin containing two thiol groups substrate was taken along with the amount of water required for the reaction, which was then tapped and given a brief spin to ensure proper mixing. After this AuNP was added to the reaction mix. The total reaction mix was then transferred to polystyrene cuvettes and the reaction was allowed to run for 30 min with a time interval of 5min. The resulting UV/Vis absorption spectra were obtained using U-3900 spectrophotometer (Hitachi). Similarly, experiments were carried out with ubiquitin lacking two terminal cysteines as negative control. In this the reaction mix was prepared as follows:

Table 4.

Chemical	Reaction Volume	Concentration
AuNP	200 μ l	50.0 μ M
Ubiquitin	15 μ l	0.25 μ M
Water	785 μ l	-
Total =	1000 μ l	-

Dispersion of Gold Nanoparticles by UCHL1 and detection by surface plasmon resonance

To induce dispersion of aggregated gold nanoparticles due to two-thiol substrate, different amounts of recombinant UCH-L1 was added to the 30nm gold nanoparticles and spectral changes were recorded. The reaction mix was prepared as described in Table 5.

Table 5.

Chemical	Reaction Volume	Concentration
AuNP	200 μ l	50.0 μ M
HS-Ubi-SH	15 μ l	0.25 μ M
UCHL1	5 μ l	1.25 μ M
H ₂ O	780 μ l	
Total=	1000 μ l	

UCHL1 was diluted following the scheme as mentioned in **Table 6**. The reaction was then kept for 2h incubation at 37°C and the required reaction volume was made to 1 ml with water. After this AuNP was added to the reaction mix. The total reaction mix (1ml) was then transferred to polystyrene cuvettes and the reaction was allowed to run for 30 min with a time interval of 5min. The resulting UV/Vis absorption spectra were recorded in U3900 spectrophotometer (Hitachi) and the observed change in wavelengths was noted.

Table 6.

Stock Dilution	Final concentration in cuvette (ng/ml)
1:10	180
1:20	90
1:40	45
1:100	18
1:1000	1.8
1:10000	1.8

Fluorescence-based UCHL-1 assay using Ubiquitin-AMC

UbAMC was dissolved water to make stock solution of final concentration of 100 μ M. Different dilution of UCH-L1 was made as mentioned in the **Table 7** and UbAMC (5 μ M) were mixed in reaction buffer (10 mM Tris•HCl, pH 8.0) was added to initiate the enzymatic reaction (300 μ L final volume). The rate of AMC cleavage was monitored at 25°C with a Molecular Devices spectrofluorometer with excitation at 340 nm and emission at 440 nm.

Table 7.

Chemical	Reaction Volume	Concentration
Ub-AMC	15 μ l	5 μ M
100mM Tris-HCl, pH 8.0	30 μ l	10 mM
UCHL1	30 μ l	see Table 8
H ₂ O	225 μ l	
Total=	300 μ l	

Table 8.

Stock UCHL1 conc. (ng/ml)	Dilution Factor	Dilution 1	Conc. in Dil. 1	Dilution 2	Concentration in cuvette (ng/ml)
A					
10 ⁷	1:1000	10 μ l UCHL1 + 990 μ l H ₂ O	10 ⁵	30 μ l of Dil-1 in 300 μ l H ₂ O	10 ⁴
B					
10 ⁷	1:10000	100 μ l of A + 900 μ l H ₂ O	10 ⁴	30 μ l of Dil-1 in 300 μ l H ₂ O	10 ³
C					
10 ⁶	1:100000	100 μ l of B + 900 μ l H ₂ O	10 ³	30 μ l of Dil-1 in 300 μ l H ₂ O	10 ²
D					
10 ⁶	1:200000	50 μ l of B + 500 μ l H ₂ O	500	30 μ l of Dil-1 in 300 μ l H ₂ O	50
E					
10 ⁶	1:400000	25 μ l of B + 500 μ l H ₂ O	250	30 μ l of Dil-1 in 300 μ l H ₂ O	25
F					
10 ⁶	1:1000000	100 μ l of C + 900 μ l H ₂ O	10 ²	30 μ l of Dil-1 in 300 μ l H ₂ O	10

Chapter 3

Results and Discussion

4.1 Cloning, Expression and purification of UCH-L1: Full length UCH-L1 was PCR amplified and examined by 0.8% agarose gel electrophoresis. The expected PCR amplification of size 672 bp was obtained. The full length *UCHL1* product and plasmid pRSETA were sequentially digested by BamHI and HindIII and ligated. The clone was selected by slower mobility compared to negative control (pRSETA). To confirm the presence of UCH-L1 and also orientation, restriction analysis of the slow moving clones was performed using enzymes *NcoI* and *ScaI*. The insert release of 1200 bp confirms the presence of UCH-L1 gene in correct orientation (**Fig. 1**).

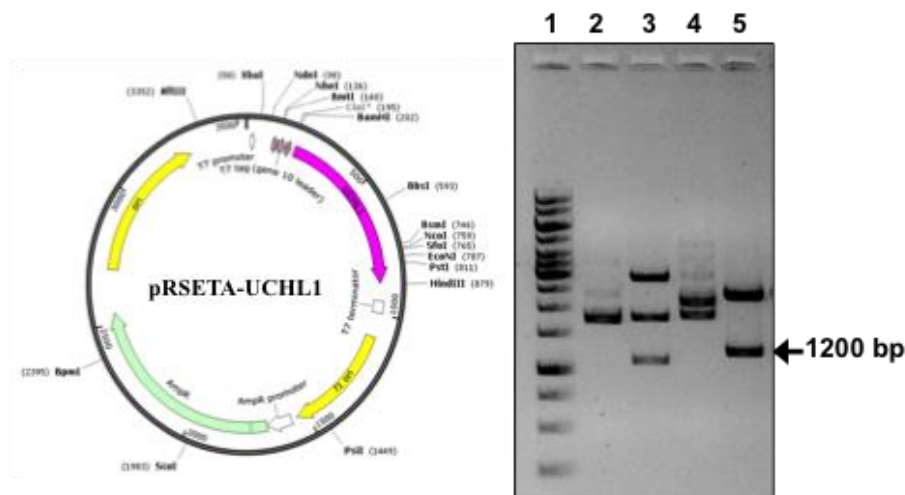


Figure 1. Restriction analysis of pRSETA-UCHL1 plasmid for conformation of positive clones. Lane 1, 1kb Dna Marker; lane 2, pRSETA vector; lane 3, *NcoI* and *ScaI* digested pRSETA vector; lane 4, pRSETA-UCHL1; lane 5: *NcoI* and *ScaI* digested pRSETA-UCHL1. *NcoI* and *ScaI* site are present within UCH-L1 gene and pRSETA-vector, respectively. Thus, digestion with these enzymes release a fragment of 1200 bp.

For recombinant protein expression, *Escherichia coli* BL21(DE3) cells were transformed with pRSETA-UCHL1. These cells were grown and induced for protein expression by IPTG. Expression of the recombinant protein was confirmed by SDS-PAGE analysis in 12% gel and staining with coomassie brilliant blue (**Fig. 2**). Since the proteins had the C-terminal his tag, they were purified by Ni-NTA affinity chromatography (**Fig. 3**).

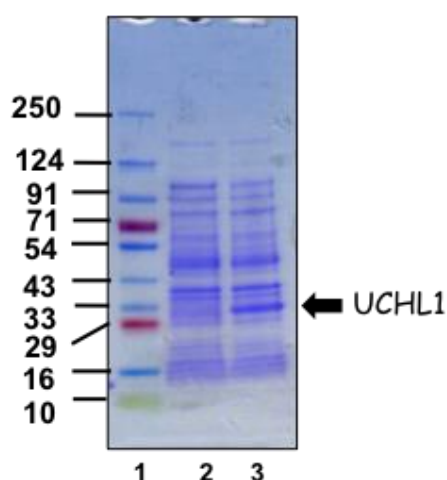


Figure 2. Expression of Recombinant UCHL1 in *E. coli*. Lane 1: Protein molecular wt marker; lane 2, total cell extract; lane 3, IPTG-Induced total cell extract showing overexpression of UCHL1

4.2 Designing of UCH-L1 substrate. We designed the peptide substrate with two salient features: (a) Two thiol-groups at the two termini of the molecule (To facilitate aggregation of gold NPs) (b) An additional lysine residue to the carboxy-terminal of 76 amino-acid ubiquitin (as UCHL1 cleaves the peptide bond between the carboxy-terminal glycine residue (Gly⁷⁶) of ubiquitin and the subsequent lysine residue (Lys⁷⁷)) [19]. In order to create the UCHL1 substrate containing two terminal thiol groups (Ub-SH), a cysteine residue (Cys⁸⁴) was added as the last residue of the substrate at the carboxyl-terminus. In order to create the other terminal thiol-groups on ubiquitin molecule, lysine at position 63 (Lys⁶³) was decided be

mutated to cystine by site-directed mutagenesis, as structure of ubiquitin clearly reveals that Lys⁶³ is located complete opposite to carboxy-terminus of ubiquitin. Thus the substrate peptide will have two cysteine thiol groups (from Cys⁸⁴ and Cys⁶³) at the head and tail position of the molecule. The C-terminal eight amino acids (containing the Lys⁷⁷, 6x His and Cys⁸³) were added to ubiquitin sequence during cloning using a specific PCR primer (**Fig. 4**).

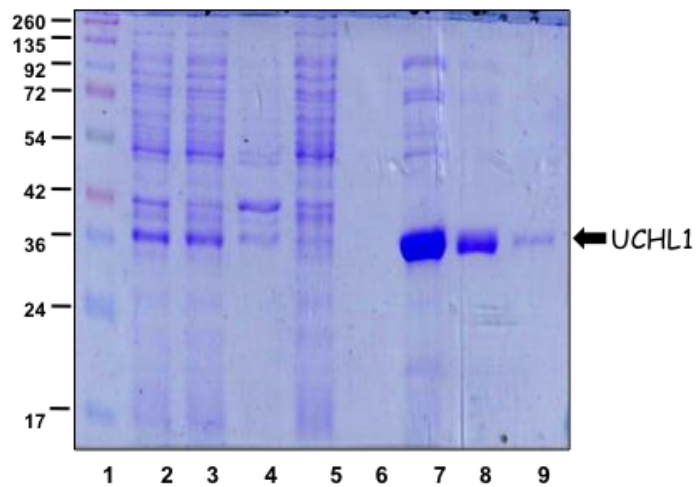


Figure 3. Affinity purification of His-tag UCHL1. Lane 1, Molecular wt marker; lane 2, total cell extract; lane 3, soluble fraction; lane 4, insoluble fraction; lane 5, unbound flow-through fraction; lane 6, wash fraction; Lane 7-9, fraction eluted with 250mM imidazole.

4.3. Cloning of Ub with C-terminal extension with single thiol group: Full length Ubiquitin was PCR amplified and examined by 0.8% agarose gel electrophoresis. The expected PCR amplification of size 255 bp was observed. The full length Ubiquitin product and plasmid pRSETA were sequentially digested by NdeI and HindIII and ligated. The entire ligation mixture was transformed to 50 µl of DH5α competent cells and grown on LB-plates containing 100 µg/ml ampicillin. After overnight incubation at 37°C, 9 colonies were selected and grown in 2ml LB-ampicillin. Further, plasmid was purified by alkaline lysis method [32]. Of the 9 colonies analyzed, 1 was moving slow compared to negative control (pRSETA). To

confirm the presence of Ubiquitin in right orientation, restriction analysis of the slow moving clone was performed using enzymes *NdeI* and *HindIII*. The insert release of 255 bp confirms the presence of Ubiquitin gene in correct orientation. For recombinant expression of Ub, *Escherichia coli* BL21(DE3)pLysS cells transformed with pRSETA-Ub. These cells were grown and induced for protein expression by 1mM IPTG. Expression of the recombinant protein was confirmed by SDS-PAGE analysis in 12% gel and staining with coomassie brilliant blue. The size of the recombinant Ub substrate was **9.63** kDa. Among the clones analyzed 1 clone was positive for overexpression of Ubiquitin (**Fig. 5**).

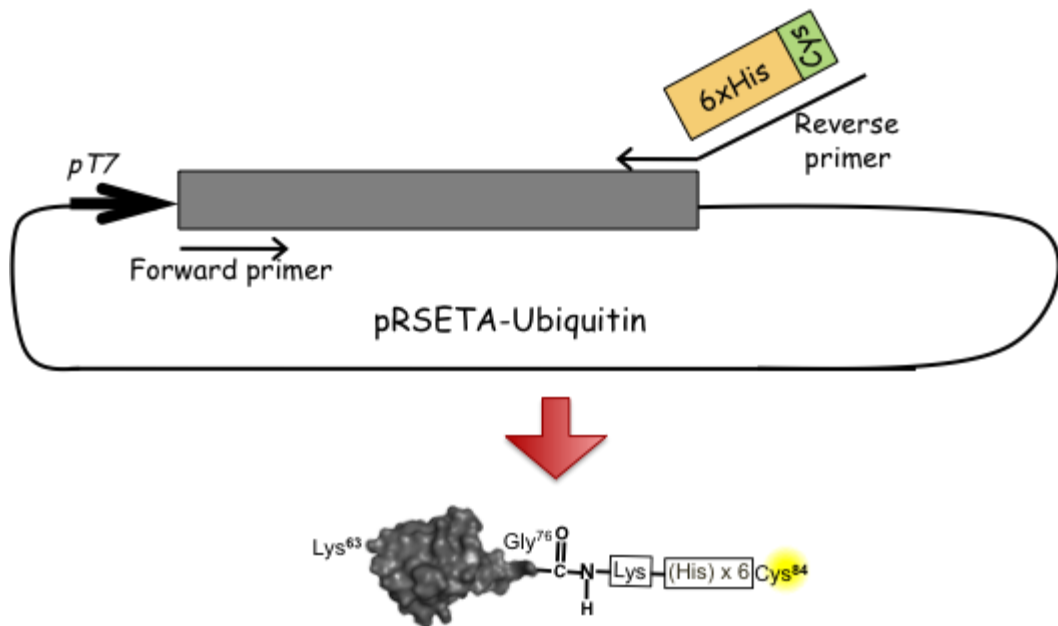


Figure 4. Schematic representation of strategy of designing UCHL1 substrate. Reverse primer was designed to contain 6-His tag and a Cys residue to generate a terminal thiol group.

4.5 Site-directed mutagenesis to generate Ub-SH: Using specific oligonucleotides, Lys63 of Ub was mutated to Cys63. The mutation was confirmed by sequencing (**Fig. 6**).

4.6. Expression and purification of Ub-SH: Recombinant Ub-SH containing Cys63 mutation and C-terminal 6-his tag was expressed in *Escherichia coli* BL21(DE3)pLysS cells and induced for protein expression by 1mM IPTG. Expression of the recombinant protein was confirmed by SDS-PAGE analysis in 12% gel and staining with coomassie brilliant blue. Expressed protein was then purified by Ni-NTA affinity purification (Fig. 7).

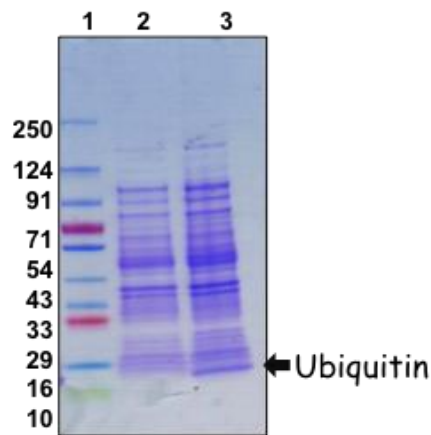


Figure 5. Expression of Recombinant Ub in *E. coli*. Lane 1:Protein molecular wt marker; lane 2, total cell extract; lane 3, IPTG-Induced total cell extract showing overexpression of Ub

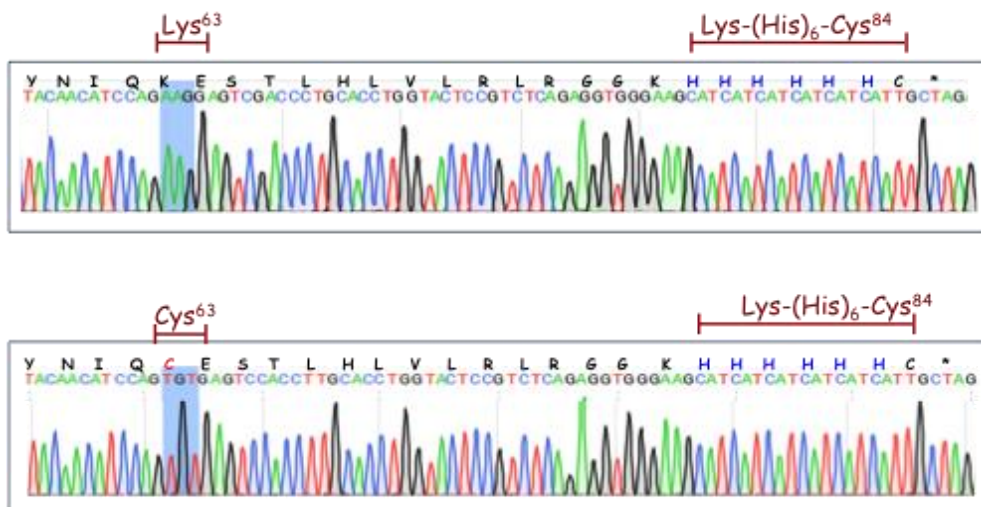


Figure 6. Sequencing results showing mutation of Lys63 to Cys63 in pRSETA-Ub

4.9 The synthesis of citrate stabilized gold nanoparticles: The gold nanoparticles (AuNPs) used in this work were synthesized by the reduction of H₂AuCl₄ with citrate. Similar procedures for gold nanoparticles synthesis has been demonstrated before. Protocols also enabled the preparation of approximately 20-40 nm spherical particles with narrow size distribution were used. Among different nanoparticles, 30nm particles were characterized using TEM, DLS and UV-Vis spectroscopy (Fig. 8).

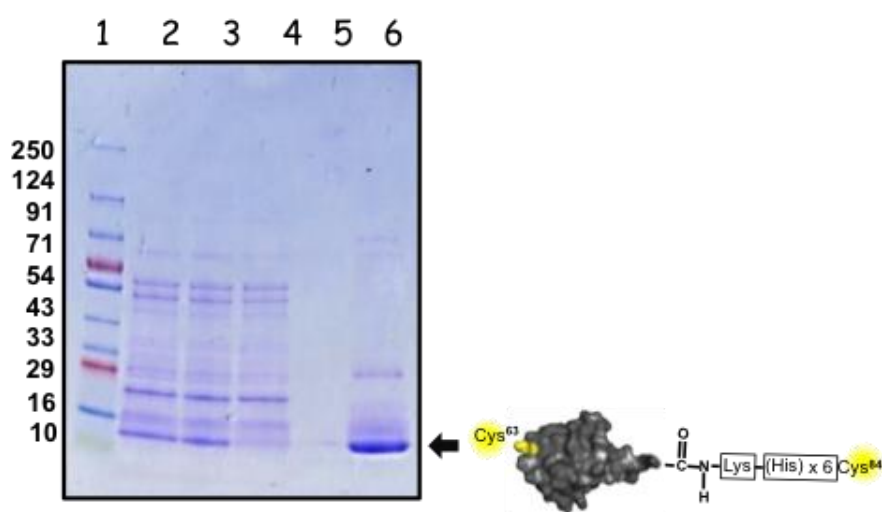


Figure 7. Purification of Recombinant Ub. Lane 1:Protein molecular wt marker; lane 2, total cell extract; lane 3, Soluble cell extract; lane 4, unbound fraction; lane 5, wash fraction; lane 6, Elution fraction.

4.10 Characterization of 30nm gold nanoparticles: The synthesized AuNPs were characterized by transmission electron microscopy (TEM) and UV-Vis absorption spectroscopy. The photograph seen in **Fig. 8** is from a sample of 30nm particles synthesized according to the procedure described in previous section. **Fig. 8** shows the TEM image of the particle; spherically shaped nanoparticles and a few triangular or ellipsoidal shaped particles could be seen. The diameter of the nanoparticles is ~30.0 nm and the plasmon absorption peak is at 529 nm. Characteristically, gold nanoparticles show surface plasmon absorption in the UV-visible absorption

spectrum. The absorption peak positions are dependent on the medium surrounding of the particles. A change in the nature of constituent surrounding ligand shell results to shift of the peak. Characterization of the prepared citrate stabilized AuNPs was done by determination of their extinction coefficients as well as observing their absorption peaks and absorption curve appearances. **Figure 9** shows the resulting absorption spectra of the analyzed AuNP samples, each having absorption maximum falling within the spectral range of 520 nm to 535 nm wavelength. Their maximum absorption peaks, A_{\max} values are summarized in **table 2**. According to the observation made in **Fig. 9**, the maximum peak shifts to longer wavelength (red-shift) with increasing particle size.

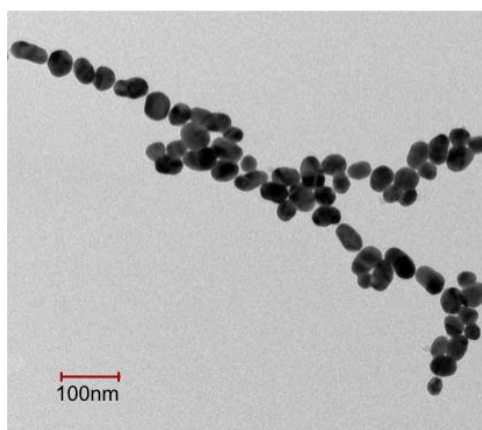


Figure 8. TEM images of gold nanoparticles.

4.9 Aggregation of gold nanoparticles using two-thiol Ub substrate: Aggregation of 30nm gold nanoparticles (0.25 mM HAuCl_4 , 1% trisodium citrate) was induced by purified two-thiol Ub substrate. To induce aggregation 15 μl of peptide (2.40 $\mu\text{g/ml}$; 0.25 μM) was added to 30nm gold nanoparticles (200 μl ; 50 μM). The aggregation of coated gold nanoparticles took 30 min to complete. However, a color change from red to purple was observed within 1-2 min. To maximize the absorption

change at 650 nm, we used an assay time of 30 min. At longer times, precipitation of large aggregates and a clear supernatant are observed and absorption intensity is no longer concentration-dependent. The UV/vis absorption spectra of solution containing coated gold nanoparticles is shown in **Figure 9**.

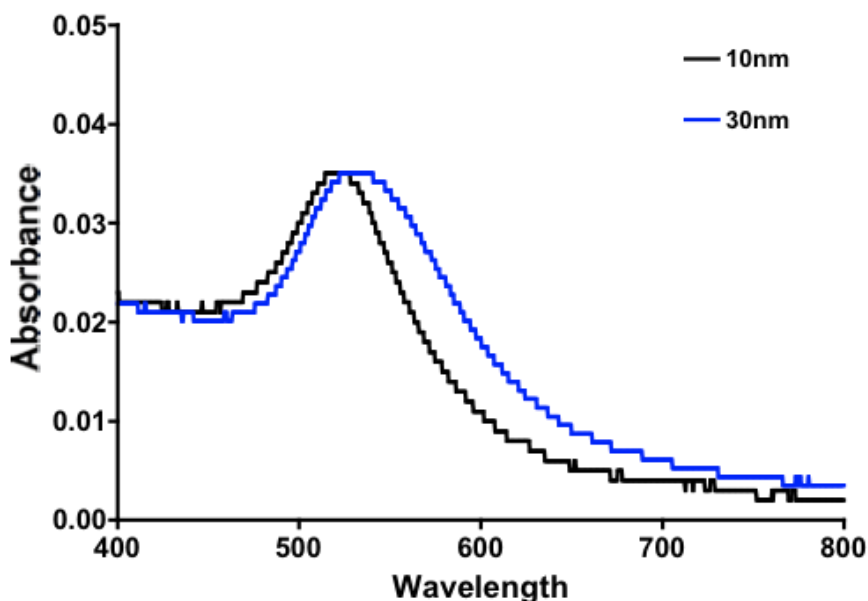
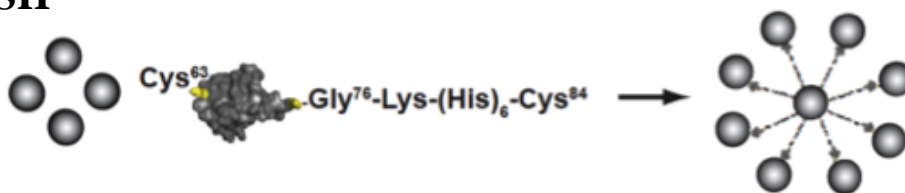


Figure 9. Absorption spectra of 10nm and 30nm gold nanoparticles.

5.0 Aggregation of Gold nanoparticle is specific. To confirm that the aggregation that we are observing is specific to gold nanoparticle, we added (15 μ l, 2.40 μ g/ml/0.25 μ M) ubiquitin substrate with single thiol group. As shown in figure **Figure 10**, no aggregation of gold nanoparticle was observed. Curve "a" shows the absorption spectrum at 650 nm of a solution containing Ub (single thiol groups). Curve "b" shows the absorption profile at 650 nm of a solution containing Ub-SH (two thiol groups). When we analyzed the time course of absorption change after adding Ub and Ub-SH peptide, we noticed that absorption intensity at 650 nm remained constant as seen from the graph at time 0 min and 30 min with Ub, indicating that aggregation did not take place. This is attributed to the lack of

multiple binding sites and the inability to form non-specific aggregates among a large numbers of particles. To test the specificity we also used Ub-SH which is cleaved by UCHL1. As shown in figure 11(a), when the peptide was added to the gold colloids, gradual change of the absorbance was obvious (Fig. 11).

Ub-SH



Ub

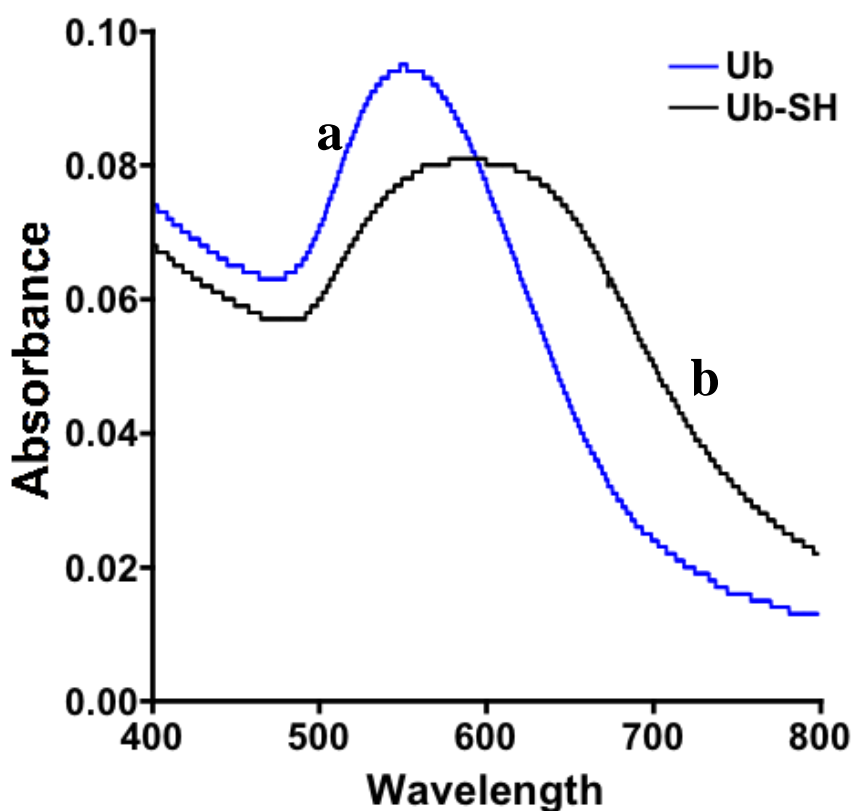


Fig 10. UV-visible absorption spectra for 30nm nanoarticle in presence of (a) Ub and (b) Ub-SH

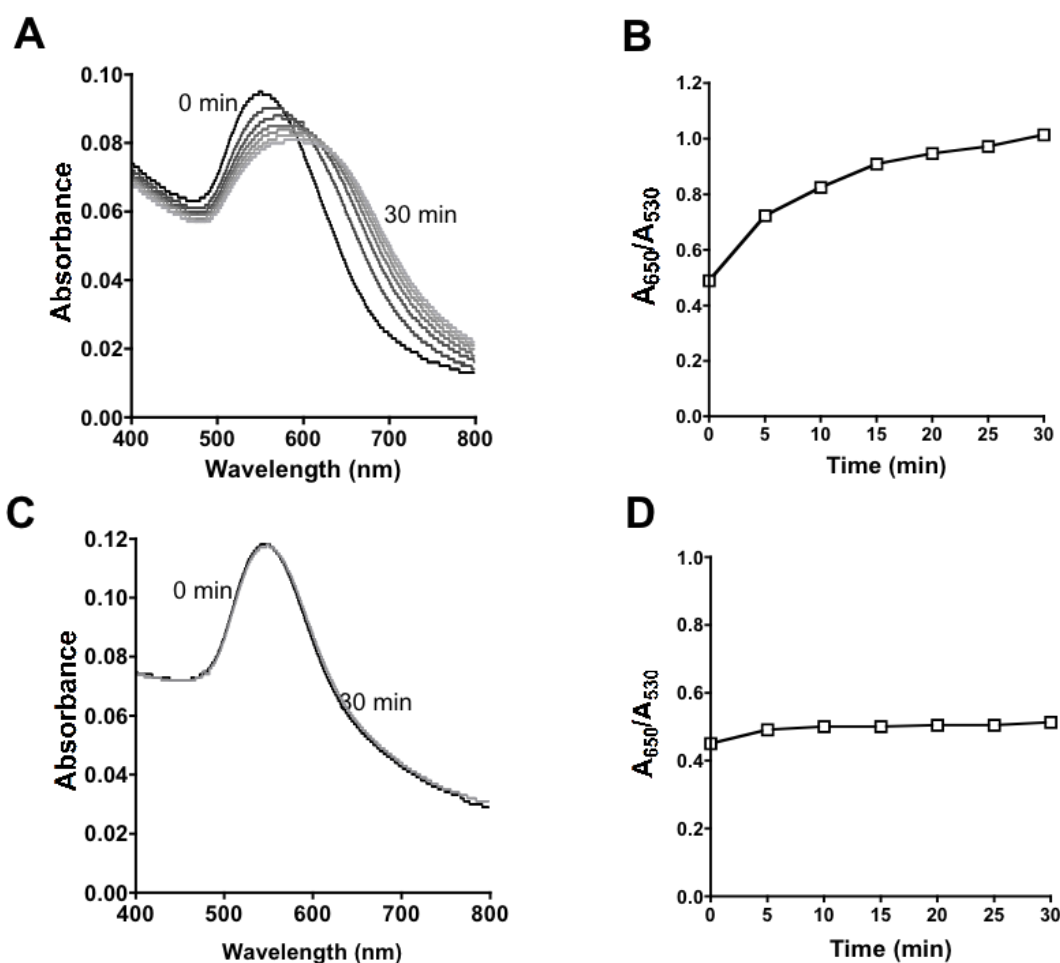


Figure 11. (A) 30 min scan of UV-Visible spectra following addition of Ub-SH to gold nanoparticles (B) Plots of the absorption ratio value from A, A_{650nm}/A_{530nm} , against time (min) (C) 30 min scan of UV-Visible spectra following addition of Ub to gold nanoparticles (D) Plots of the absorption ratio value from C, A_{650nm}/A_{530nm} , against time (min)

4.11. The dispersal of gold nanoparticles using recombinant UCH-L1. To induce dispersion of aggregated gold nanoparticles due to two-thiol substrate, recombinant UCH-L1 was added to the nanoparticle solutions and spectral changes were recorded as shown in **Figure 12**. Interestingly, the color changes were obtained in the presence of **5 μ l UCH-L1 (1.25 μ M/38 μ g/ml)** range only. The spectral and colorimetric changes were obtained immediately when higher concentration of

UCHL1 was added to the gold nanoparticle solution, due cleavage of the peptide substrate.

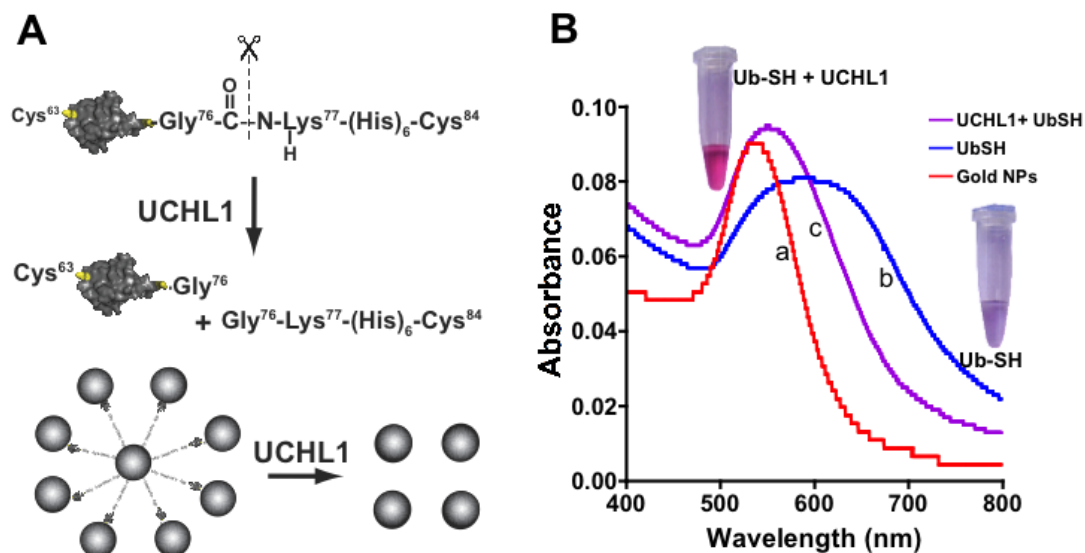


Figure 12. (A) Schematic representation of UCHL1 assay. (B) UV-Visible spectra of 30nm gold nanoparticles (a) in presence of Ub-SH (b) and Ub-SH + UCHL1 (c)

4.12. Sensitivity of AuNP-based assay using thiol containing UCHL1 substrate and recombinant UCHL1. To increase the sensitivity of AuNP-based assay using thiol containing substrate and recombinant UCHL1, the concentration of the gold nanoparticles was increased from 0.25mM to 0.50mM. Then, to induce dispersion of aggregated nanoparticles, recombinant UCHL1 was added to aggregated nanoparticle solution at various concentrations and the absorbance spectrum for each sample was scanned between 400 and 800 nm. The 650/530 nm ratio (A_{650}/A_{530}) was then plotted against UCHL1 concentration (**Fig. 13**). The concentration of UCHL1 used in these reactions (1.8-180 ng/ml UCHL1) were close to the amount of UCHL1 released in the blood serum following brain injury. To assess sensitivity of this gold nanoparticle based assay we also performed Ub-AMC based UCHL1 assay. **Figure 14** shows UCHL1 catalysed hydrolysis of Ub-AMC at several

concentration of UCHL1. UCHL1 catalysed hydrolysis of Ub-AMC releases AMC which showed emission at 440nm. When we plotted UCHL1 concentration against free AMC fluorescence, we detected similar UCHL1-dependen hydrolysis of Ub-AMC (Fig 15). Therefore, novel gold nanoparticle based assay described here, can be used to detect functional UCHL1 *in vitro* via a hydrolysis of a short peptide containing ubiquitin with similar sensitivity in comparison with presently available fluorescence-based assay.

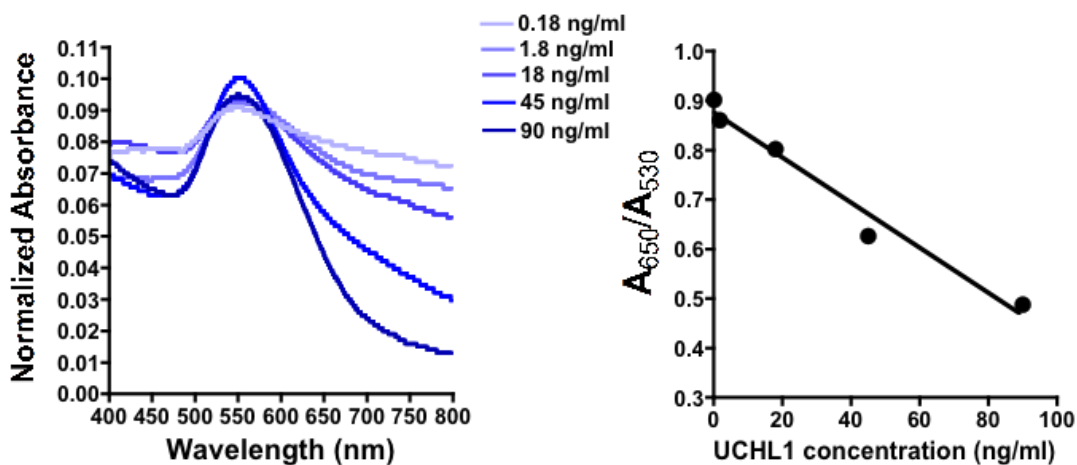


Figure 13. Dispersion of Ub-SH aggregated gold nanoparticles is proportional to UCHL1 concentration.

The relevance of this nanoparticle-based assay includes that it does not require nanoparticle functionalization or any specific instrumentation; simple colorimetric analysis qualitative detection of UCHL1 that can be performed unlike fluorescence based assay where Ub-AMC has two critical disadvantages. First, AMC has an excitation wavelength in UV range, thus exciting at 240 -360nm is known to excite a significant number of screening compounds and thus will generate a large fraction of false positives. Second, AMC is covalently attached to C-terminal COOH of

ubiquitin and not via a e-NH₂ group as found under physiological conditions (41). Therefore, the nanoparticle-based assay could be used for the preliminary analysis of biological fluids without referring to specialized laboratories. Nevertheless, it provides a sensibility comparable to that provided by the Fluorescence-based assay.

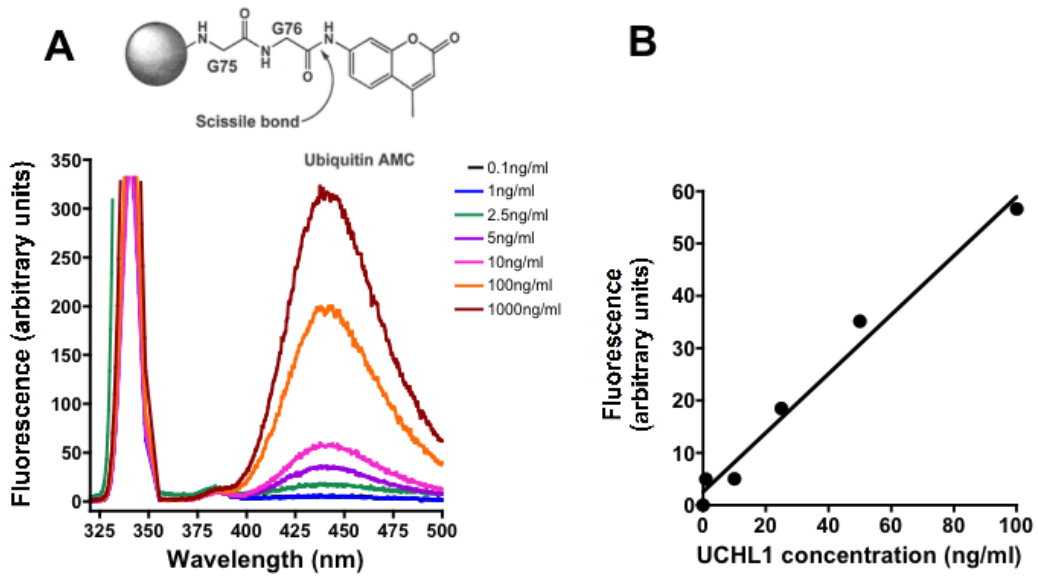


Figure 14. Hydrolysis of Ub-AMC is proportional to UCHL1 concentration.

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