Materials and Methods
Hapten Design and synthesis

Molecular Modeling
Monoacetyl morphine (MAM), with a molecular weight of 328 Da, was conjugated to a well known antigenic carrier protein BSA. The preparation of carrier protein (BSA)-hapten (MAM) conjugate required the presence of reactive functional groups on both the protein and hapten molecules. MAM does not have any functional group that can be used for conjugation with carrier protein. Therefore, acidic groups (-COOH) were chemically introduced into MAM molecule without changing the major salient features of the molecular structure. The hydroxyl group attached to the phenyl ring in MAM was derivatized to generate a site for the attachment of protein having reactive functional group at the other end.

For this, molecular models of the target compound (MAM) and the proposed hapten structure (MAM-COOH) were generated using the Program Argus Lab 4.0.1 software package. The potentials were assigned using the ‘Molecular Mechanics Hamiltonian’ forced field and the structures were subjected to energy minimization using the “GADock engine”. The ball and stick models were used for the comparison of spatial structure of the hapten. Steric parameters such as volume, surface area, total negative charge and polarizability of the molecules were determined using the Hyperchem software (Singh et al., 2010).

The initial designing of hapten structure was carried out using the ChemDraw utility in the ChemOffice software. Subsequently the 3D-structure of MAM was saved in PDB (Protein Data Bank) format. It was ensured that most of the structural features of MAM were retained in the proposed hapten structure (MAM-COOH).
Synthesis of MAM-COOH from MAM

Reagents
Monoacetylmorphine (MAM) was obtained from Sigma Chemical Co., USA. Chloroacetic acid, sodium hydroxide, acetonitrile were obtained from Merck. All other chemicals used were AR grade.

Chemical synthesis was done by the following method:
Monoacetyl morphine derivative (3-hydroxy-6-acetyl-(5α, 6α)-7, 8-didehydro-4, 5-epoxy-17-methylmorphinan) (MAM–COOH) was synthesized by chemical modification of the 3-OH group to 3-COOH group of MAM. For reaction mixture of 1.0 ml, 3 μM of MAM and 24 μM of chloroacetic acid were mixed slowly in 45 μM sodium hydroxide and 30 μM acetonitrile. The mixture was reflux heated for 3 h at 90 °C in an inert nitrogen atmosphere. The completion of the reaction was confirmed by TLC (Rf value approximately 0.13) using hexane: chloroform: triethylamine (9:9:4) as solvent system, and by FTIR spectroscopy (Perkin-Elmer).

Purification
The final derivatives were purified using the chloroform: methanol (9:1) as solvent on silica gel (60-120 mesh) column and purification was confirmed by the band position in TLC on silica gel 60 pre-coated Merck F254 TLC sheets and the spots were observed under short UV (Rajnanda et al., 1985).

Characterization
UV spectroscopy
The UV absorption spectra were taken in the range of 200-300 nm for all the opiate drugs (i.e. Heroin, Morphine and Codeine), MAM and its derivative MAM-COOH at a concentration of 1.0 mg/ml in acetonitrile using a Shimadzu UV-1601 UV-visible spectrophotometer. The base line was corrected using acetonitrile before taking the spectra.
Infra Red Spectroscopy (IR)

FT-IR spectra of the MAM and MAM-COOH was recorded on FT-IR spectrometer (Perkin Elmer, Spectrum BXII) using an ATR cuvette. A thin film of the protein sample was coated on ATR cell in an inert environment. The spectra were recorded in the range of 4400-400 cm\(^{-1}\) and used for structural analysis by curve fitting method. Samples were mixed with potassium bromide (KBr) at concentration of 5% w/w. First of all the baseline was adjusted for all the curves and 5 point averaging was used to smoothen FT-IR data. The solid state IR was recorded by making pellets of the mixture at 10-15 tons in hand hydraulic press. The pellets were placed in window cell provided by the manufacturer and the IR spectra were recorded. The spectra for the background were also obtained and the final spectra for the compound were saved after adjusting for the background. Peak fitting was done using the multiple peak-fitting module of the Origin 5.0 software until good agreement was reached between the experimental curve and the sum of all the Gaussian peaks used for the generation of theoretical curve. The best Gaussian peaks that gave good fit for the experimental curve were assigned the secondary structural elements, according to previous studies done by Byler and Suzi (1986) and Karen and coworkers (1999).
Bioconjugation

Chemicals and Reagents
Standard heroin, morphine, and codeine samples were provided by the Central Forensic Science Laboratory (CFSL), Chandigarh (India). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), sulfo N-hydroxysuccinimide (sulfo-NHS), 0.2 M borate buffer pH 8.2, bovine serum albumin (BSA), ovalbumin (OVA), KLH (keyhole lymphet heamocyanin), TNBS (2, 4, 6-trinitrobenzene 1-sulfonic acid), Sinnapinic acid, TFA (Trifluoroacetic acid), Sodium periodate, ethylene diamine were purchased from Sigma Chemical Co. USA. Q Sepharose and Sepharose-4B were procured from Amersham Biosciences, India. BCA protein estimation kit was obtained from Pierce. ELISA plates were purchased from NUNC, Denmark. P10 gel filtration columns were purchased from Pharmacia, Sweden. All other chemicals were of analytical grade.

Preparation of Carrier Protein-Hapten conjugates

Activation of Hapten
The synthesized hapten (MAM-COOH) was conjugated with carrier proteins (BSA, OVA, and KLH) using EDAC and sulfo-NHS. For this, stock solution of hapten was prepared by adding 50 µM MAM-COOH in 1 ml of 0.2 M borate buffer, pH 8.6 along with 75 µM EDAC and 75 µM sulfo-NHS (Gourlaouen et al., 1998). The activation of reaction mixture was carried out for 1 h at room temperature, followed by overnight incubation at 4 °C, and then centrifuged for 10 min at 10000 g to remove the urea precipitates and the filtrate was taken as 50 µmol/ml of activated hapten solution.

Conjugation of Carrier Protein with Hapten
BSA, OVA and KLH were used as carrier proteins for conjugation with the MAM-COOH throughout in this study. The protein solution was prepared in borate buffer (0.2 M, pH 8.2) at concentration of 10 mg/ml. A fixed amount of protein (for BSA-0.15 µmol; for OVA-0.23 µmol) in 1 ml borate buffer, was reacted with variable amounts of activated hapten, in
a final volume of 1.3 ml, to prepare conjugates with variable hapten densities per molecule of the carrier protein. The supernatant, containing the activated MAM–COOH, was used to prepare the conjugate with protein by adding different concentrations of hapten (3.0, 6.0, 12.0, 15.0 and 30.0 μmol) to a fixed amount of BSA (0.15 μmol) in final volume of 1 ml, so as to prepare different molar ratios (1:20, 1:40, 1:80, 1:100 and 1:200) of BSA: MAM–COOH corresponding to MP1 to MP5, respectively (Gourlaouen et al., 1998).

Similarly, OVA-MAM conjugates were prepared at different molar ratios of 1:40, 1:80 and 1:100 corresponding to MP1 to MP3, respectively. For KLH: MAM-COOH conjugate only one ratio (1:400) was used, as the molecular weight of KLH is very high (4.5 × 10^6 ~ 1.3 × 10^7) and due to its property of precipitation. Because of its size, KLH often suffers from poor water solubility. While this may not affect its immunogenicity, it caused the difficulty in handling of KLH in solution and affected the process of conjugation. Even following removal of insoluble particles from KLH solution, it was difficult to determine the exact amount of KLH present in the solution because of its property of precipitation.

Different conjugates of BSA-MAM-COOH were prepared according to Table 1. The activated hapten-protein reaction was carried out at RT for 2 h, followed by an overnight incubation at 4°C. After the reaction was completed, the precipitated protein fraction was separated from the soluble fraction by centrifugation at 10000 rpm for 30 min. The conjugate was separated from free hapten by passing through a P10 gel filtration column (Pharmacia, Sweden).
Table 1: Showed the contents of reaction mixtures, for preparation of BSA-MAM-COOH conjugates, using different molar ratios

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Amount of protein (Vol in ml)*</th>
<th>Amount of Hapten (Vol in ml)**</th>
<th>Molar Ratio (Protein: Hapten)</th>
<th>Borate buffer added to reaction mixture (ml)</th>
<th>Total Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.15 µmole (1 ml)</td>
<td>0 (0 µl)</td>
<td>1:0</td>
<td>600</td>
<td>1.3</td>
</tr>
<tr>
<td>2.</td>
<td>0.15 µmole (1 ml)</td>
<td>3.0 µmole (60 µl)</td>
<td>1:20</td>
<td>540</td>
<td>1.3</td>
</tr>
<tr>
<td>3.</td>
<td>0.15 µmole (1 ml)</td>
<td>6.0 µmole (123.4 µl)</td>
<td>1:40</td>
<td>366</td>
<td>1.6</td>
</tr>
<tr>
<td>4.</td>
<td>0.15 µmole (1 ml)</td>
<td>12.0 µmole (240.677 µl)</td>
<td>1:80</td>
<td>359</td>
<td>1.6</td>
</tr>
<tr>
<td>5.</td>
<td>0.15 µmole (1 ml)</td>
<td>15.0 µmole (300 µl)</td>
<td>1:100</td>
<td>300</td>
<td>1.6</td>
</tr>
<tr>
<td>6.</td>
<td>0.15 µmole (1 ml)</td>
<td>30.0 µmole (600 µl)</td>
<td>1:200</td>
<td>0</td>
<td>1.6</td>
</tr>
</tbody>
</table>

Purification of BSA-MAM by Ion Exchange Chromatography

The separation of BSA-MAM-COOH from free protein was done by anion exchange chromatography (Amersham Biosciences, India). The column (Q-Sepharose, bed volume 2 ml) was equilibrated with 20 mM Tris-HCl, pH 8.0. The conjugate, prepared at different molar ratios, was loaded onto the column separately and eluted at a flow rate of 0.5 ml/min using 0–1.0 M NaCl gradient in 20 mM Tris-HCl, pH 8.0. The fractions having the maximum protein concentration as determined by OD$_{280}$ (Hitachi, U2800 UV spectrophotometer) were pooled and subjected to dialysis against 1 x PBS, pH 7.4 overnight at 4°C, with frequent changes to remove any traces of the unreacted hapten (free MAM-COOH).
Characterization of Hapten-Protein conjugates

Estimation of Protein concentration by Bicinchoninic Acid method (BCA)

The hapten-protein conjugate were prepared at different concentrations and the complex formed was quite linear with increased protein concentration over a working range of 5-2000 µg/ml of protein. The development of color did not stop even after one-hour incubation but was sufficiently slow to estimate concentration in large number of samples. The concentration of all the conjugates prepared was determined using the Pierce BCA protein estimation kit according to the manufacturer’s instruction in microwell plate format at 562 nm.

Estimation of amino groups in Protein-Hapten conjugates by chemical (TNBS) (2, 4, 6-trinitrobenzene 1-sulfonic acid) method

To 100 µl of standard solutions (0.025-1.0 mM) of L-Lysine and Glycine, added 400 µL of 0.1 N NaHCO3 and mixed well and kept under shaking conditions at room temperature for 30 min. 250 µl of 0.01% TNBS solution was added to the mixture and incubated for 2 hours at 37°C. The reaction was stopped using 250 µl of 10% SDS and 125 µl of 1 N HCL. The color developed was read at 335 nm. OD335 obtained after reacting Glycine was subtracted from the OD335 obtained after reaction of L-Lysine with TNBS. The plot between resultant OD335 and concentration gave the standard curve for quantitative estimation of the ε-amino groups. Different conjugates were prepared at concentration of 1 mg/ml and were reacted with 0.01% TNBS solution under alkaline conditions to determine the % of –NH2 groups used during conjugation. 100 µl of conjugate solution was taken and color was developed as for the standards. The difference between the OD335 of control and conjugate gave the amount of –NH2 groups used during conjugation of BSA to the MAM-COOH molecules (Habeeb et al., 1966).

Mass spectrometric methods (MALDI-TOF)

The mass of the carrier protein (BSA) and conjugates (BSA-MAM-COOH) was determined using a MALDI-TOF mass spectrometer (Kratos Analytical Systems). 3, 5-dimethoxy-4-hydroxycinnamic acid (Sinapinic acid) was prepared at a concentration of 15 mg/ml in acetonitrile. Protein conjugates dialyzed against DDW water was used at a
concentration of 0.05 mg/ml. The conjugate sample and matrix solution was mixed in equal amounts (1 µl each) and 0.5 µl of 0.1% TFA solution was added to it. The solution was prepared in situ on the stainless steel MALDI probe plates and allowed to dry at room temperature. The data was acquired with 50 shots/sample in linear mode at 30 kV and the spectrum was analyzed using the software provided with the system.

Electrophoresis

The Native-PAGE of the conjugates (different BSA-MAM-COOH molar ratio) was performed using Bio-Rad electrophoresis apparatus (Miniprotein-3) according to method developed by Laemmli (1970). The solutions for Native-PAGE were prepared by diluting 1:1 in Native Sample Buffer (10 mM Tris pH 8.0, 30 % glycerol v/v, 0.01 % bromophenol blue w/v) to final concentration of 1 mg/ml. The solutions were mixed well and kept at RT for 10 min before loading. Gels were loaded with 5 µg of sample and run for 90 min at 120 V. Coomassie blue staining was done for 2 h at room temperature, followed by destaining for 1-2 h. The gel was kept in Milli-Q water. The gel image was captured using Amersham Pharmacia Gel Doc System (Image Master VDS).

Absorption Spectroscopy

Control protein (BSA, OVA and KLH) and its respective protein-hapten conjugates were prepared at concentration of 1 mg/ml and absorption spectra for various conjugates were recorded in the 200-400 nm range using a UV-visible spectrophotometer (Shimadzu, UV-1601).

Fluorescence Spectroscopy

Control protein (BSA and OVA) and its respective protein-hapten conjugates were prepared at a concentration of 0.025 mg/ml and fluorescence spectrum was taken in the 200-400 nm range, using Shimadzu fluorescence spectrophotometer (RF-5301 PC). The excitation was given at 280 nm and emission was checked at 340 nm. The slit width was used at 5 nm.
CD (Circular Dichroism) Spectroscopy

The CD spectra of the control (BSA) and BSA-MAM-COOH conjugates were recorded at a concentration of 0.05 mg/ml in the range of 190-340 nm using a CD spectrometer (JASCO 700). The molar ellipticity ‘θ’ obtained from the CD spectrometer was converted to mean residual ellipticity ‘(θ)’ using the following relations:

\[
(θ) = \frac{θ}{10 C_r l}
\]

where \(θ\) = Ellipticity (mdeg), \(C_r\) = Mean residue molar concentration (mole/Liter), and \(l\) = Path length of the cell (cm). The \(C_r\) for control BSA and conjugates was calculated as follows:

\[
C_r = n \times C_p = n \times 1000 C' \times \frac{M_p}{M_p}
\]

where \(n\) = number of constructed residues of the macromolecule,
\(C_p\) = Molar concentration of the molecule (mole/L),
\(C'\) = Weighing weight of the macromolecule (g/ml),
\(M_p\) = Molecular weight of the polymer.
For BSA, \(n = 583\), \(C' = 0.1 \text{ mg/ml} = 0.0001 \text{ g/ml}\)
\(M_p\) = mass as determined using MALDI-TOF.

The data corrected for the actual molecular weight of the conjugates, as mean residual ellipticity was fed as input to the CDSSTR software (Johnson et al., 1999) for determination of secondary structure from CD spectrum obtained for various conjugates. The software is freely available (Sreerama and Woody, 2000) on the internet as part of the CDPRO software package from http://lamar.colostate.edu/~sreeram/CDPro/.
Labeling of Bioconjugates

Chemicals and Reagents

FITC and Gold chloride were purchased from Sigma Chemical Co. USA. Sephadex-G25 were procured from Amersham Biosciences, India. All other chemicals were of analytical grade.

Preparation of Labeled Conjugates

Preparation of Fluorescein isothiocyanate (FITC) – anti-MAM IgG antibody

A 2 mg/ml solution of anti-MAM IgG antibody was prepared in 0.025 M carbonate buffer pH 9.6. 1 mg/ml FITC was prepared in DMSO (dimethyl sulfoxide). For preparation of FITC-antibody conjugate, 50 μl of FITC solution was added dropwise into antibody solution in dark chamber at 4°C. The reaction was carried out at 4°C overnight (Roederer et al., 2004). After completion of the reaction, unreacted protein was removed initially by passing through Sephadex G-25 column and further by dialyzing the FITC-antibody conjugate against 10 mM Tris pH 8.2 and stored at -20 °C until use.

\[
\text{IgG} = 3.1 \times \frac{A_{495}}{[A_{280} - 0.31 \times A_{495}]}
\]

F: P values of 3-10 are probably optimal for any partciular IgG.

Preparation of colloidal gold- anti-MAM IgY antibody conjugate

[a] Preparation of colloidal gold solution

Monodispersed 15-20 nm colloidal gold was prepared by slight modification of Frens (1973) method. In brief, 200 ml solution of 0.01% tetrachloroauric acid (gold chloride) in double distilled water was taken in a 500 ml Erlenmeyer flask and covered with tin foil. The solution was brought to boiling point on hot plate. 8 ml of 1% trisodium citrate solution was rapidly added to the boiling gold chloride solution. The solution was allowed
to boil for 10 min till it reached typical bright wine red color of colloidal gold. The solution initially evolved into grey-purple color that turned into dark purple and finally developed into bright wine red color. Once this color was formed any further heating did not produce any change in the size of colloidal gold.

The average particle size of colloidal gold was determined using spectrophotometer at 530 nm (Shimadzu 1601A), transmission electron microscope (Hitachi Model H-7500) operated at 120 kV after negative staining of gold-antibody complex and also by zeta particle size analyzer (Delsa™ Nano, Beckman Coulter). The solution of citrate gold nanoparticles were passed through 0.2 μm filter and particle size was analyzed by particle size analyzer. TEM sample was prepared by placing drop of colloidal gold on carbon coated copper grid and measurement was done at operating voltage 100 kV.

[b] Determination of Optimum pH and Optimum Protein amount for Conjugation

Colloidal gold was conjugated to protein at predetermined optimum pH and using optimum amount of protein required for maximum stabilization of colloidal gold. The optimum pH was determined as follows:

In different vials the pH of colloidal gold solution was adjusted at steps of 0.5 pH units starting from pH 5.0 to 10.0 by using 0.1 N HCl and 0.2 N K₂CO₃ for adjustment of pH. At each pH unit the colloidal gold was allowed to react with excess protein and the stabilization was checked by adding 100 μl of 10% NaCl per ml of the colloidal gold solution. The unstabilized gold gets flocculated in presence of excess electrolyte and gave a blackish purple color while stabilized gold solution retained their wine red color. The minimum pH at which the color of the colloidal gold did not show any change was taken as the optimum pH for conjugation to target protein.

Further the optimum amount of protein required for maximum stabilization of colloidal gold was determined by taking different amount of protein 1 to 100 μg in different wells of a microtiter plate, the final volume was equalized using deionized water. In each well, 100 μl of colloidal gold solution at optimum pH and was allowed to be stabilized for 15 min. As done earlier adding excess electrolyte i.e., 10 μl of 10% NaCl solution in each well and observing the change in color for the stabilization. The minimum amount of
protein that was able to prevent the flocculation of colloidal gold was taken as the optimum amount of protein required for maximum stabilization of gold at optimum pH.

[c] Preparation of Colloidal Gold antibody Immunoprobe

To a pre-calculated amount of protein, the required amount of colloidal gold solution was added and adjusted to optimum pH, with constant stirring. The stabilization was allowed to proceed for 15-20 min. For preparing antibody-gold conjugate probe, 90 μg of anti-MAM IgY antibodies, prepared in phosphate buffer (20 mM, pH 7.4), was added drop-wise into 1 ml colloidal gold solution ([Au] = 2.4 x 10^{-4} mol/L) under mild stirring condition to reach final antibody concentration of 90 μg/ml. The pH of the colloidal gold solution was maintained at 7.4 by addition of diluted 10 mM Na₂CO₃ before adding antibody. The mixture was incubated overnight at 4 °C and centrifuged at 12000 rpm for 30 min to remove unconjugated antibody from the solution. The protein stabilized colloidal gold appeared as loose pellet at the bottom of the centrifuge tube while unstabilized colloidal gold particles made shiny dark colored spot at the side of the centrifuge tube. Remove the supernatant without disturbing the loose pellet. The pellet was washed three times with 10 mM Tris (pH 8.0) containing 3% BSA to remove traces of free antibody or loosely bound protein conjugate molecules. Finally the pellet was resuspended in the 2 ml phosphate buffer (20 mM, pH 7.4) containing 20% glycerol v/v and 0.02% sodium azide as preservative and stored at 4°C for regular use. The final concentration of colloidal gold in the antibody-gold conjugate solution was about 4.8 x 10^{-4} mol/l and antibody concentration was 0.21 μg/ml. A Hitachi 2800 UV-vis spectrophotometer was used to measure the absorbance of gold nanoparticles and antibody labeled gold nanoparticles. The gold-antibody complex was negatively stained with PTA (Sodium (Potassium) phosphotungstate) stain for the visualization of protein coating on the surface of gold nanoparticles. For this, the antibody-gold complex was treated with 2% aqueous solution of PTA stain (equal volume) and place a drop of this mixture onto grid and leave for ~20 sec. Remove almost all the solution with filter paper and air dry (Zhang et al., 2010) for visualization by TEM. Further, the size of gold nanoparticles was measured by Zeta Particle size analyzer after filtration of gold nanoparticles and gold anti-MAM IgY antibody complex.
Antibody Generation and its characterization

Chemicals and Reagents
Bovine serum albumin (BSA), ovalbumin (OVA), keyhole lympet hemocyanin (KLH), complete Freund’s adjuvant (CFA), incomplete Freund’s adjuvant (IFA), goat anti-rabbit/mouse IgG-HRP, goat anti-chicken IgY-HRP, Isotypes (mouse monoclonal isotyping kit) were purchased from Sigma Chemical Co. USA. Standard heroin, morphine, and codeine samples were provided by the Central Forensic Science Laboratory (CFSL), Chandigarh (India). Protein-A Sepharose and HiTrap IgY were procured from Amersham Biosciences, India. 3,3',5,5'-tetramethylbenzidine (TMB) was purchased from Bangalore Genei, India. 0.22 μm filters, nitrocellulose membrane were obtained from Advanced Molecular Devices, Ambala Cantt., India. ELISA plates were purchased from NUNC, Denmark. Ethanolamine, sodium acetate buffer, pH 4.5, CM5 dextran chip, EDC and NHS were procured from Amersham Biosciences. HPLC grade water was purchased from Merck. All other chemicals were of analytical grade.

Immunization and sera collection in rabbits and mice
Two groups of young female New Zealand white rabbits (8-10 weeks old) and BALB/c mice (8-10 weeks) were immunized. Each rabbit (250 μg) and mice (25 μg) was immunized subcutaneously with immunizing antigen (BSA-MAM conjugate) prepared as 1:1 emulsion in Freund’s Complete Adjuvant (FCA). Further boosters were given at an interval of 21 days using 1:1 emulsion of antigen in Freund’s Incomplete Adjuvant (FIA). Blood was collected from the rabbit/mice after every 5th day onwards at alternate days after the booster dose was given. The blood was allowed to clot for one hour at room temperature. The clot thus formed was punctured and allowed the serum to separate overnight at 4 °C. Serum was collected after centrifugation for 30 min at 10000 rpm and pooled. The serum was kept at 56 °C for 20 min followed by keeping in ice immediately. The antibody titer in the pooled serum was determined using ELISA assay (Hudson and Hay, 1989).
**Immunization and egg collection in chicken**

Young 8-10 weeks old white female leghorn chicken was immunized intramuscularly with 150 µg of BSA-MAM conjugate in the breast muscle emulsified with FCA followed by three booster injections given in FIA at an interval of 21 days. The antibodies were harvested from egg yolk. The eggs were collected after every 5th day of 1st booster and stored at 4 °C until use.

**Purification of antibodies using affinity chromatography**

**IgG**

[a] Ammonium sulfate (SAS) precipitation

The whole IgG of serum proteins from the antiserum was precipitated using Saturated Ammonium Sulfate (SAS) (761 g/l, pH adjusted to pH 7.0) precipitation method (Hudson and Hay, 1989). In brief, the antiserum was centrifuged at 20000 rpm for 30 min to remove any cellular debris. To the serum supernatant, added an equal volume of SAS slowly while stirring to achieve a final concentration of 50% ammonium sulfate and kept the mixture at 4 °C overnight with constant stirring. The solution was centrifuged at 10000 rpm for 30 min and the precipitates were resuspended in minimum amount of PBS after removing the supernatant. The protein solution was dialyzed against PBS containing 0.01% sodium azide, overnight at 4 °C with frequent changes of dialysis buffer. The dialyzed protein solution was centrifuged at 10000 rpm for 30 min and the protein concentration was determined at OD$_{280}$.

[b] Protein-A affinity purification

The SAS precipitated anti-MAM IgG serum was diluted to final concentration of 5 mg/ml and used for loading on the protein-A sepharose column. The anti-MAM IgG serum was passed through the column twice, flow through was collected and the column was washed till OD$_{280}$ of the washing buffer (PBS, pH 7.4) fall down to a minimum of 0.03 or lower. The elution was done using minimum amount of elution buffer i.e., glycine-HCl (50 mM, pH 2.5). The buffer coming out of the column was monitored for pH change and the
fractions were collected as soon as the pH started dropping. 1 ml fractions were collected, till the OD<sub>280</sub> fell down to base line. The collected fractions were neutralized immediately with 50 µl of 0.1 M Tris, pH 8.0 and kept at 4°C (Hudson and Hay, 1989). The fractions with maximum protein were pooled and dialyzed against PBS overnight at 4°C with frequent changes. The concentration of the purified antibodies was determined at OD<sub>280</sub>. Purity of IgG was checked by Native-PAGE. For IgG fragmentation, 2 mg/ml anti-MAM IgG antibody was transferred into 0.2 M sodium acetate buffer (pH 4.5) by dialysis and equal volume of pepsin was added to the anti-MAM IgG solution. Final w/w ratio of pepsin to antibody was 1:100 and incubated at 37 °C for 4 h. At the designated time, 2 M Trizma Base was added to adjust the pH to neutral and stop the reaction. Finally, the products of each reaction were analyzed on non reducing SDS-PAGE (Hudson and Hay, 1989).

**IgY**

[a] **Potassium sulfate (SPS) precipitation**

The IgY antibodies were purified according to the manufacturer’s instructions (Amersham Biosciences). The yolk was separated carefully and washed with deionized water. The yolk sac was disrupted by inserting the needle and the contents were allowed to dip through a nylon mesh into measuring cylinder. The sample preparation was done by taking 1 part of egg yolk and 9 part of distilled water. The suspension was incubated for 6 h at 4 °C. The supernatant containing IgY was collected by centrifugation at 10000 rpm for 25 min at 4 °C to precipitate the lipids. While stirring slowly, added potassium sulfate to final concentration of 0.5 M followed by adjusting the pH at 7.5. The sample was then filtered through 0.45 µm filter immediately before applying to the Hitrap IgY purification HP column.

[b] **HiTrap IgY affinity purification**

The anti-MAM IgY purification was done by Hitrap IgY column (as described in the guidelines provided by Amersham Biosciences). Briefly, the column was equilibrated with 5 bed volume of binding buffer (20 mM sodium phosphate + 0.5 M K<sub>2</sub>SO<sub>4</sub>, pH 7.5). The sample was then applied on to the column and washed ten times with the binding buffer to
remove the unbound protein until OD at 280 became zero. The bound anti-MAM IgY was then eluted by elution buffer (20 mM sodium phosphate, pH 7.5) and dialyzed against PBS, pH 7.4 at 4 °C with subsequent changes and stored at -20 °C until further use. Native-PAGE was run to check the purity of antibody.

**Characterization of antibodies and labeled conjugates**

**Dot-Blot Assay**

Antigen (BSA-MAM) (for checking antibody reactivity) was coated on the nitrocellulose membrane at 1μg/μl concentration in carbonate buffer (50 mM, pH 9.4) and allowed it to air dry. The membrane was washed with PBS twice and nonspecific binding sites were blocked using PBS containing 10% skimmed milk for 1 h at 37°C. The membrane was washed with PBST thrice and once with PBS, followed by incubation with anti-MAM IgG and IgY primary antibodies at different concentrations (1:1K to 1:20K) for 2 h at 37°C. After incubation, the membrane was again washed three times with PBST and finally with PBS. For checking the reactivity of antibodies, bound primary antibodies were detected by using HRP-labeled Goat anti rabbit/chicken antibodies. Incubation with secondary antibodies was carried out for 1 h at 37°C at predetermined dilution of 1:10000. Colour was developed using TMB/H$_2$O$_2$ substrate for localization. The dot blot photographs were captured using a Pharmacia Gel Doc system (Image Master VDS).

**Isotyping of anti-MAM IgG (mice) antibodies:**

The assay was done according to the Isotyping kit protocol provided by the SIGMA. In brief, 5 μg/ml BSA-MAM (100 μl/well) was coated on 96 well Elisa plate (Nunc, Denmark) in carbonate buffer (50 mM, pH 9.6) and incubate overnight at 4°C. After washing thrice with phosphate buffer saline (PBS, pH 7.4) the non-specific binding sites were blocked with 5% defatted skimmed milk (200μl/well) in PBS and incubated for 1h at 37°C. Plates were washed thrice with PBST and finally with PBS. 100 μl/well anti-MAM IgG antibodies solution prepared in PBSM (containing 0.1% defatted skimmed milk), was added at various dilutions as required and incubated for 1 h at 37°C. Isotypes (IgM, IgG2a, IgG2b, IgG3, IgG1, IgA) (1: 1000 in PBST + 0.1% BSA) were added separately and incubated at 37°C for 1 h. Plates were washed thrice with PBST and
finally with PBS. Add goat anti-mouse HRP (1:10,000) in PBST + 0.1% BSA. The plates were thoroughly washed three times with PBST and once with PBS. Plates were incubated for 1 h at 37°C followed by washing as in earlier step. TMB/H$_2$O$_2$ (100 μl/well) was added and incubated for 15 min to develop a color. The blue color developed was stopped after 15 min using 50 μl/well 1N H$_2$SO$_4$ as stop solution. The blue color is converted to bright yellow color. The absorbance was measured at 450 nm using a BioTek micro plate ELISA reader.

**Affinity binding studies using BIACore**

[a] **Coupling reaction of BSA-MAM to CM5 dextran gel**

The carboxymethylated dextran (CM-dextran) matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride) prepared in ultrapure water and injected the mixture over the sensor chip surface for 7 min at flow rate of 5 μl/min. The BSA-MAM conjugate to be immobilized (100 μg/ml) was dissolved in 10 mM sodium acetate buffer, pH 4.5, and injected over the surface for 40 min at a flow rate of 5 μl/min. The unreacted sites on the sensor chip surface were then capped by injection of 1M ethanolamine prepared in ultrapure water pH 8.5, for 7 min (Brennan et al., 2003).

[b] **Sample preparation for sensor analysis and regeneration conditions required**

The anti-MAM IgG antibody was diluted in HBS buffered saline solution (HBS, pH 7.4) at a concentration 100 μg/ml. All buffers and solutions used were made up using ultrapure water, degassed and sterile filtered. Regeneration conditions for the removal of the polyclonal antibody from the surface of the chip were optimized and found to require a 1 min pulse of 10 mM Glycine-HCl, pH 3.0.

**Indirect ELISA**

BSA-MAM was coated on 96 well Elisa plate (Nunc, Denmark) at a concentration of 5 μg/ml in carbonate buffer (50 mM, pH 9.6) by adding 100 μl/well and incubate
overnight at 4°C. After washing twice with phosphate buffer saline (PBS, pH 7.4) the non-specific binding sites were blocked with 5% non fatty skimmed milk in PBS using 200 μl/well and incubating for 2 h at 37°C. Plates were washed thrice with PBS containing 0.05% Tween-20 (PBST) and finally with PBS. 100 μl/well of anti-MAM IgG antibodies solution (from rabbit) or antisera (to check the antibody titer from rabbit) and anti-MAM IgY antibodies from egg yolk prepared in PBS containing 0.1% defatted skimmed milk was added at various dilutions, respectively and incubated for 2 h at 37°C. The plates were thoroughly washed three times with PBST and finally with PBS. HRP-labeled rabbit anti-IgG/ chicken anti-IgY was used as secondary antibody at dilution of 1:10000 and added 100 μl/well, respectively. Plates were incubated for 1 h at 37°C followed by thorough washing as in earlier step to remove any nonspecifically bound secondary antibody. Color was developed using TMB/H2O2 as substrate (100μl/well). The blue color developed was stopped after 15 min using 50 μl/well 1N H2SO4 as stop solution. The absorbance was measured at 450 nm using BioTek micro plate ELISA reader (Dankwardt et al., 2000).

Competitive Inhibition ELISA for IgG and IgY
For competitive assays, ELISA plates were coated with 100 μl of BSA-MAM conjugate (5 μg/ml) prepared in carbonate buffer (20 mM, pH 9.6) and incubated overnight at 4°C. The plates were subsequently washed with PBS (50 mM, pH 7.4). 10% defatted skimmed milk was used for blocking in PBS for 1 h at 37°C. The ELISA plates were washed with PBS containing 0.05% Tween-20 (PBST) and once with PBS and were subjected to competitive inhibition immunoassay by mixing 100 μl of standard drug solution (heroin, MAM, morphine or codeine) with equal volume of anti-MAM IgG antibody (1:1000 in PBS) and anti-MAM IgY antibody (1:1000 in PBS), respectively. The standard drug solutions were prepared in PBS at concentrations between 0.01-1000 ng/ml for IgG and 1.0 to 1000 ng/ml for IgY, respectively. The plates were incubated for 2 h at 37°C and washed thoroughly with PBS. 100 μl of secondary antibody solution (for IgG-goat anti-rabbit IgG-HRP and for IgY goat anti-chicken IgY-HRP) was added into each well (1:10000 in PBS) and plates were incubated for 1 h at 37°C. Subsequently, 100 μl of substrate (TMB) was added into each well. The enzymatic reaction was carried out in dark for 15 min at room temperature. The reaction was stopped by adding 50 μl of 1N H2SO4 into each well, followed by
measurement of absorbance at 450 nm using the ELISA plate reader (Biotek- XS Plus) (Dankwardt et al., 2000).

The extent of antibody binding inhibition was measured from comparison of the OD at known concentration of antigen and zero concentration of antigen. The readings from different assays were normalized using the following equation:

\[
\%B/B_0 = \frac{(A-A_{ex}/A_0-A_{ex})}{(A_0-A_{ex})} \times 100
\]

where,

- \( B \) = O.D. at known concentration of antigen
- \( B_0 \) = O.D. at zero concentration of antigen

The \( B/B_0 \) values obtained for each known concentration were plotted against the concentration of antigen to prepare a standard inhibition curve for the antigen detected. \( A, A_0 \) and \( A_{ex} \) were the absorbance intensities of antigen, antigen at zero concentration, and antigen at excess concentration, respectively.

The % reactivities of related analogues were calculated on the basis of standard calibration curves in the range of pg/ml to ng/ml level. After normalizing the data by \( %B/Bo \) transformation, the specific hapten concentration yielding 50% inhibition was used to calculate the % reactivity according to the formula:

\[
\% \text{ Reactivity} = \frac{H}{C} \times 100
\]

where \( H \) and \( C \) are concentrations of standard hapten and cross reacting hapten/analogue at 50% \( B/Bo \) respectively (Beatty et al., 1987).

**Determination of affinity constant of anti-MAM IgG and IgY antibodies**

For the measurement of antibody affinity constant (\( K_{asf} \)), microtiter plates were coated with antigen (BSA-MAM) at different concentrations (5, 2.5, 1.25 and 0.625 μg/ml) prepared in carbonate buffer. After washing and blocking with 5% defatted skimmed milk in PBS, 100 μl of anti-MAM IgG antibody solution (0.1 to 1000 ng/ml in PBS) and anti-MAM-IgY antibody (1.0 to 1000 ng/ml) was added into each well separately and plates were incubated for 2 h at 37 °C. After incubation with secondary antibody (For IgG HRP labeled anti-rabbit
and for IgY HRP labeled anti-chicken antibody, respectively) and TMB treatment, as described above, absorbance was measured at 450 nm. $K_{aff}$ values were determined based on the law of mass action (Beatty et al., 1987) using the following relation:

$$K_{aff} = \frac{(n-1)/2 \{n[Ab'] - [Ab]\}}{n = [Ag]/[Ag']},$$

where,

$[Ag]$ and $[Ag']$ are two different coated antigen concentrations, and $[Ag']$ and $[Ag]$ are the observed anti-MAM IgG antibody concentrations at 50% of the maximum absorbance value.

**Competitive fluorescence inhibition immunoassay**

The anti-MAM IgG antibody was labeled with fluorescein isothiocyanate (FITC) according to the method described earlier (Goding et al., 1976). The BSA-MAM coated (5µg/ml) ELISA plates were subjected to competitive fluorescence immunoassay by adding 100 µl of each standard drug solution (heroin, MAM, morphine, or codeine) in wells and mixing with equal volume of anti-MAM IgG–FITC solution (1:100 in PBS). The standard drug solutions were prepared in PBS at concentrations ranging between 0.01 to 100 ng/ml. The plates were incubated for 2 h at 37 °C. After washing, the fluorescence intensity of each well was measured by fluorescence plate reader (Molecular Devices, USA) at 495 nm excitation and 525 nm emission wavelengths.

Analysis of the competitive inhibition assay data was performed by normalizing the fluorescence values using the following formula:

$$\%F/F_0 = \frac{F-F_{ex}}{F_0-F_{ex}} \times 100$$

Where $F$, $F_0$, and $F_{ex}$ are the fluorescence intensities of sample, hapten at zero concentration, and hapten at excess concentration, respectively.

% reactivity of related analogues was calculated on the basis of standard calibration curves in the pg/ml to ng/ml range. The results were normalized by $F/F_0$ transformation and the
specific antigen concentration yielding 50% inhibition was used to calculate the % reactivity by:

\[ \text{% Reactivity} = \frac{H}{C} \times 100 \]

where H and C are concentrations of standard hapten and cross reacting hapten/analogue at 50% F/Fo respectively (Beatty et al., 1987).

**Stability Assays**

**Indirect Fluorescence Immunoassay with different stabilizers for IgG**

Hapten specific antibodies (anti-MAM IgG antibodies) were generated by immunizing young New Zealand white rabbits with standard immunization and purification protocols as described earlier (Hudon and Hay, 1989). The antibodies were characterized by the fluorescence based ELISA (enzyme-linked immunosorbent assay) method. For this, ELISA plates were first coated with 5 μg/ml conjugated hapten (BSA-MAM) prepared in carbonate buffer (pH 9.4) and kept for incubation overnight at 4 °C. After washing twice with 0.01 M Tris-Cl (pH 8.5) containing 9 g/l NaCl (washing solution A), blocking was done with 5% skimmed milk in 0.1 M NaHCO₃ (pH 8.5) by incubating the plate for 2 h at 37 °C. Rabbit anti-MAM IgG antibody of different concentrations in 0.01 M Tris-Cl (pH 8.5) containing 10 g/l BSA, was added into each well of the ELISA plate and incubated for 2 h at 37 °C. The plate was washed again and subsequently, 100 μl of FITC labeled goat anti-rabbit antibody (1:10000 dilution) was added into each well of the plate and kept for incubation at 37 °C for 2 h. The plates were washed as previously, add 30% glycerol in 0.05 M carbonate buffer, pH 9.2, and incubated for 5 min at 37 °C. Glycerol solution was discarded and emptied wells were incubated for 15 min at 37 °C. The fluorescence counts were measured using the Fluorescence Microtiter Plate Reader (BioTek synergy 2, Finland) facilitated with an excitation filter at 495 nm and an emission filter at 525 nm (Petrou et al., 2007).

In case of control, the wells were not treated with glycerol after completion of the assay. Before finalizing glycerol as a stabilizer, standardization was done by using different stabilizers for the selection of best stabilizing agent. For this, prepare different concentrations (10 %, 20%, 30% and 40%) of PEG 6000 and glycerol in 50mM carbonate buffer pH 9.2 and incubated at different time period viz. 0, 15, 30, 45, 60 and 90 min.
CD (Circular Dichroism) Spectroscopy of IgG and IgY at different temperature and time points

For CD spectrum studies using anti-MAM IgG and IgY antibodies, same concentrations (0.1 mg/ml) for both the antibodies were taken and heated at different temperatures viz. 4 °C, 37 °C, 45 °C, 60 °C. The spectra were taken at different time intervals viz. 0 h, 2 h, 4 h and 8 h respectively. The change in the % helical content (α and β helix) was calculated using a CD spectrometer (JASCO 700).

ELISA at different time points

The binding studies were done to check the stability of antibodies at higher temperature and also time factor was considered. Therefore, to perform this task, ELISA assay was done to check the binding of antibody (IgG and IgY type) separately at different temperatures and at different time intervals same as for the CD measurements. In brief, BSA-MAM was coated on 96 well Elisa plate (Nunc, Denmark) at a concentration of 5 μg/ml in carbonate buffer (50 mM, pH 9.6) by adding 100 μl per well and incubate overnight at 4°C. After washing twice with phosphate buffer saline (PBS, pH 7.4) the non-specific binding sites were blocked with 5% defatted skimmed milk in PBS using 200 μl/well and incubated for 2 h at 37°C. Plates were washed thrice with PBS containing 0.05% Tween-20 (PBST) and finally with PBS. The anti-MAM IgG and IgY antibodies, were pre-incubated at different temperatures viz. 4 °C, 37 °C, 45 °C, 60 °C and at different time intervals 2 h, 4 h and 8 h. 100 μl/well prepared in PBS containing 0.1% defatted skimmed milk, was added at various dilutions as required and incubated for 2 h at 37°C. The plates were thoroughly washed thrice with PBST and finally with PBS. HRP labeled anti-IgG/IgY was used as secondary antibody at dilution of 1:10,000 and 100 μl/well was added into each well. Plates were incubated for 1 h at 37°C followed by a thorough washing as in earlier step to remove any nonspecifically bound secondary antibody. Color was developed using TMB/H₂O₂ as substrate (100μl/well). The blue color developed was stopped after 15 min using 50 μl/well 1N H₂SO₄ as stop solution. The absorbance was measured at 450 nm using a BioTek micro plate ELISA reader.
Development of Immunobiosensor and its characterization

Chemicals and reagents

Rabbit and chicken anti-MAM antibodies, coating antigens OVA-MAM, BSA-MAM, and anti-MAM IgG FITC-antibody (FITC-IgG Ab) and colloidal gold labeled anti-MAM IgY antibody conjugate (Au-IgY Ab) were prepared as described earlier. ELISA plates were purchased from Nunc, USA. Goat anti-rabbit IgG-HRP conjugate were purchased from Sigma, St. Louise, USA. TMB/H$_2$O$_2$ ready to use substrate were purchased from Bangalore Genei, India. The Nitrocellulose membrane laminates and plastic cassettes for housing flow through assembly were purchased from Advanced Microdevices, Ambala, India. Carboxylated SWCNT powder was purchased from Cheap Tubes Inc., Singapore. Sodium dodecyl sulfate (SDS) surfactant and phosphate buffer saline (PBS, pH 7.4) solution were purchased from Sigma Aldrich. All other chemicals were analytical reagent grade and purchased locally.

Development of strip based immunochromatographic biosensor for MAM

The cassette consisted of a nitrocellulose membrane (15 μm pore size) laminated on a plastic base. In contact with the membrane was conjugate release matrix in which colloidal gold particles are embedded. A sample pad that was in close contact with the release matrix provided the site for application of buffer/sample. As soon as the buffer/sample were loaded in the well, the liquid started moving and the colloidal gold reagent embedded in the matrix is released in presence of fluid. Once the fluid reached the membrane it moves forward aided by the capillary action of the membrane.

Coating of antibodies on the nitrocellulose membrane

The OVA-MAM conjugate was coated on the nitrocellulose membrane (15 μm) using an easy line printer provided by Advanced Microdevices, Ambala Cantt., India. The amount of conjugate coating was controlled by the speed of the moving coater over the membrane surface. A fine needle disposes of the conjugate solution as the coater moves from end to end.
end on the membrane. 3% methanol was added to the coating solution to get better adsorption of the conjugate. After coating, non-specific binding sites on the laminates were blocked using various blocking agents such as 5% skimmed milk, 5% BSA (bovine serum albumin), 5% sucrose, 5% PVA (polyvinyl alcohol), 5% PVP (polyvinyl pyrrolidone) in phosphate buffer (PB) (20 mM, pH 7.4) etc. Afterwards the coated laminates were dried immediately in a laminar flow and fit into the plastic cassette (Kaur et al, 2007 and 2008).

**Embedding of colloidal gold in release matrix**

The colloidal gold reagents were embedded in the release matrix using dip and dry method. The strips of released matrix were dipped in a predetermined dilution of colloidal gold reagent in PB containing 5% BSA, 5% sucrose and 5% PVP. On an average 1 ml colloidal gold solution was sufficient for embedding release matrix for use with 45-50 strips.

**Strip based immunochromatographic assay for detection of MAM**

A lateral flow dipstick kit was developed by using an application pad made of glass fiber membrane and a signal generation test line on nitrocellulose (NC) membrane pad on which OVA-MAM conjugate was immobilized (detection zone). A cellulose membrane was used as an adsorption pad. Test line was made up with 2.0 mg/ml OVA-MAM conjugate prepared in 3% methanol and dispensed on NC membrane (2.0 µl/line) using an Easy Printer (Advance Microdevices, Ambala, India). The remaining area of NC membrane was blocked with a mixture of 5% BSA, 5% sucrose and 5% PVP in PB (20 mM, pH 7.4) for 1 h at RT. The sample pads having glass fiber lining were soaked in anti-MAM IgY-gold conjugate solution (1:400). The combined strips (sample pad, NC membrane and absorption pad) were dried at RT in a clean air chamber (Laminar hood) for 2 h and inserted in plastic cassettes for their application as dipstick device. Analysis of drug samples (standard in the range of 0.01-1000 ng/ml and real urine samples) was done by adding 50 µL sample prepared in 20 mM phosphate buffer (pH 7.0) containing 1% Tween 20 into the sample well of the dipstick. The reaction of the analyte and anti-MAM IgY-gold probe took place immediately at the conjugate pad area. The analyte and OVA-MAM conjugate coated on to a test line compete their binding with anti-MAM IgY-gold probe. The intensity of color developed (reversibly) due to the presence of tracer, correlates with the amount of analyte.
present in the sample and the intensity of signal generated at the test line was measured by a

gel documentation system (Cami Imager™ Ready, Alpha Innotech Corporation) for the

semi-quantification of analyte to calculate the %B/B₀ and IC₅₀ values as described earlier in

competitive ELISA protocol for IgY (Xiulan et al., 2005; Kaur et al., 2007).

**Development of liquid gated-field effect transistors (LG-FET) for the biosensing of MAM**

**Preparation of CNT suspension**

To increase the carboxylic functional groups for better molecule attachment, 20 mg of

purchased carboxylated CNT material was acid treated in 100 ml, 3:1 volume ratio of

concentrated H₂SO₄ and HNO₃ and refluxed overnight at 50 °C. The refluxed solution was

centrifuged several times to separate the tubes from acid solution and neutralize the

solution until pH 6.0. To further enhance tube dispersity, the 1 mg/ml treated solution

was diluted 10 times with DW to 0.1 mg/ml concentration and 1% w/v SDS was added

into the final volume. The suspension was sonicated, followed by centrifugation for

1 h at 14000 rpm to remove the non-dispersed bundles. The extracted supernatant was

kept as a stock solution for the entire experiment (Koehne et al., 2004).

**Characterization of SWCNTs**

**Molecular Modeling**

In simulating the BSA-SWCNT interaction with Argus Lab 4.0.1, the native conformation of

the BSA structure was obtained from SAM-T08 server (Katzman et al., 2008). The

geometry of SWCNT was built in Arguslab 4.0.1 and optimized through universal force

field molecular mechanics Hamiltonian. Optimized geometry was obtained when the

difference between two calculated Hamiltonian converged to a value less than 0.1 kcal/mol.

The best interaction configuration was searched through GADock engine, which performed

minimization of the binding free energy, and the extent of the interaction was quantified by

measuring the size of the binding pocket, as well as calculating the dipole moment of the

BSA, before and after the interaction.
AFM (Atomic force microscopy)

Pristine SWCNTs were used as purchased from Carbon Solution, Inc. Carboxylated SWCNTs were synthesized from pristine nanotubes through acid treatment (Liu et al., 1998). The physisorption of the BSA on to the nanotube was characterized by AFM Nanoscope IIIa tapping mode in ambient environment on bare poly dimethyl siloxane substrate. The substrate material was chosen because its typical roughness (root mean square 0.772 nm) was not exceeded the diameter of the SWCNTs (2–4 nm).

Fluorescence and CD (Circular Dichroism) Spectroscopy

The stability of the conjugate, as well as changes in the BSA structure, was monitored by fluorescence spectroscopy (Perkin Elmer LS 50B, excitation: 290 nm) and CD spectroscopy (Jasco-J810, 0.1 cm path length quartz cell), employed to study the BSA conformation upon interaction with SWCNT. Pristine and carboxylated SWCNTs were incubated in aqueous BSA solution (100 μg/ml, 2h) with concentrations ranged from 0, 0.01, 0.02, 0.025, 0.03 and 0.05 mg/ml (or 0, 1.74, 3.74, 4.34, 5.21 and 8.68 x 10^-9 M, respectively), prepared in 20 mM phosphate buffer pH 7.0. % α-helix content in the BSA was calculated from the resulting CD spectra as detailed elsewhere (Wangoo et al., 2008).

SWCNT-LGFET fabrication

A facile method was employed to fabricate a low-cost, all-plastic SWCNT LGFETs. The device architecture simplified the fabrication process greatly because it was made from only two materials: PDMS and SWCNT, and it did not require photolithography process, except for the mold fabrication.

Microchannels were first fabricated in PDMS by casting a 10:1 ratio of uncured resin and curing agent in a silicon master mold. SWCNT films were prepared by vacuum filtration from the above-mentioned SWCNT suspension (Shim et al., 2002). Tuning the sheet resistance, $R_s$, was facilitated by controlling the filtration volume hence, the density of the nanotube network films. Film with $R_s$ larger than 300 kΩ/sq, which was used as the transistor channel, was transferred to a flat PDMS; whereas, thick film with $R_s$ less than 1 kΩ/sq, which was used for source and drain contact pads, was transferred to the PDMS
substrate carried the microfluidic channel. Stamping the thick film to PDMS carrier with microfluidic channel (400 μm) defined automatically the SWCNT source and drain electrodes, and also the channel length (L) of the transistor. The lamination process was completed by bringing together the two PDMS carriers. By injecting electrolyte solution into the microchannel, a typical transfer characteristic can be obtained from the SWCNT LGFET.

**Optimization of sensing response**

To achieve consistent immunosensing response with minimized false signal, series of background works were conducted to ensure reliable biomolecules attachment and process optimization through systematic layer-by-layer analysis. Influential parameters such as pH and ionic strength of electrolyte solution, were optimized to determine the appropriate condition for biomolecule’s activity for best binding efficacy. Three different pH ranges 4.5, 7.4 and 9.6 were selected at two different molarities 0.5 M and 0.05 M concentrations of phosphate buffer solution.

As an electrostatically sensitive device, one of the important parameters that influence the device sensitivity was the Debye length \( \lambda_{D} \). Upon bio-analyte absorption onto CNT network, the counter ions in the electrolyte solution accumulated near the bio-analyte, caused the electrostatic potential of the absorbed analyte to decay over a length due to the shielding effect which can be represented by the following equation:

\[
\lambda_{D} = \left( \frac{\varepsilon_0 \varepsilon_r kT}{2N_A e^2 l} \right)^{1/2}
\]

Where, \( I \) is the ionic strength of the electrolyte in mol/m\(^3\), \( \varepsilon_0 \) is the permittivity of free space, \( \varepsilon_r \) is the dielectric constant, \( k \) is the Boltzmann’s constant, \( T \) is the absolute temperature in Kelvin, \( N \) is Avogadro’s number and \( e \) is the elementary charge.

In addition to this, different blocking agents (10% PEG, skimmed milk, PEG+skimmed milk, and Tween-20, respectively) were also used for effective blocking and to prevent non-specific binding.
MAM sensing

In our earlier study on protein–CNT submitted to interaction carboxylated single-walled CNT (SWCNT) was shown to interact better with BSA (Wijya et al., 2009). Hence, the acid-treated carboxylated SWCNT prepared using the above mentioned treatment was used as the active channel of the LGFET. The carboxylic groups were first activated with EDC and sulfo-NHS for 1 hour at room temperature, rinsed with 50 mM PB solution (pH 9.5), followed by 10 μg/ml BSA-MAM injection and overnight incubation at 4°C. Excess unbound molecules were removed by rinsing the microchannel with copious of PB solution and the device were ready for target anti-MAM IgG detection.

Electrical measurement

Electrical measurement of the CNT-LGFET was performed using a home-built LabView system with the testing protocol similar to the reported literature (Heller et al., 2008; Minot et al., 2007). For real time monitoring, a liquid gate potential (Vg) at -0.5 V was applied to the electrolyte through reference electrode (3M KCl, FLEXREF, World Precision Instruments) and a small drain bias (Vd) of 10 mV applied over the source and drain electrodes to obtain the kinetic response at respective sensing steps.

Standardization of immunoassay using different approaches

In the first approach, anti-MAM IgG-Ab was incubated directly onto SWCNT channel for one hour, followed by morphine injection to observe the signal change. For the second approach, an enhancement technique was employed by detecting BSA-MAM conjugate rather than MAM. The last approach reversed the protocol where the BSA-MAM conjugate was first immobilized onto the CNT network, followed anti-MAM IgG-Ab injection. For this scheme, in order to improve binding efficiency of BSA-MAM conjugate to the SWCNT network, EDC and sulfo-NHS were used to activate the carboxylic group of the SWCNT prior to the addition of conjugate molecules. In addition, blocking step using 10% v/v Tween-20 in PBS was introduced into the channel after the immobilization for the second and third approach, to prevent any non-specific binding.
Selection of blocking agent was carried out in previous study where three candidates, i.e. polyethylene glycol (PEG), Tween-20 and skimmed milk, were incubated for 1 hour on the SWCNT network, followed by Bovine serum albumin (BSA) injection and the change in the conductance was recorded.

Two electrical measurements were performed with a home-made system. A current pre-amplifier (Stanford Research System SR-570) and a multi-channel voltage source module (National Instrument) were connected to a stage manipulator. Two channels of the voltage source module were employed to supply the source-drain (V_{DS}) and gate bias (V_{G}). These two source channels were also branched to two readout channels in the module for monitoring purposes. The current pre-amplifier was installed to read the source-drain current (I_{DS}) level. The current pre-amplifier and the voltage source modules were synchronized to a PC with the aid of a programming code written in LabVIEW 7.1.

The measurement system was able to perform I_{DS}-V_{G} characterization as well as kinetic measurements. In the former, the V_{G} was swept across a predetermined voltage window (-800 to -200 mV) to a reference electrode (3M KCI Flexref, World Precision Instrument), and an ultra low-frequency small signal AC (V_{max} 10 mV) bias was employed for the V_{DS}. The I_{DS} was then determined as the root-mean-square value of the readout from the current pre-amplifier, and the leakage current (I_{leak}) was taken from the average of this same current readout. The I_{DS} was then plotted against V_{G} to show the I_{DS}-V_{G} behavior of the transistor. Meanwhile, in kinetic mode, the system applied a constant value of V_{G} and the similar ultra low-frequency small signal AC bias for the V_{DS}. The I_{DS} current was then plotted against time to probe the kinetic behavior of the LGFET.

Degradation analysis after administration of heroin in Mice

Preparation of standard solutions

All stock and test solutions of heroin, MAM, morphine and codeine were prepared (1mg/ml) in methanol and stored at -20°C when not in use. To avoid the chemical hydrolysis, standard solutions were prepared within 30 min of use. To stabilize the heroin,
we added acetate buffer immediately to thawed samples. The solution was vortex mixed for 10 sec, centrifuged for 10 min at 10000 rpm and filtered with 0.2 µm filters.

**Experimental Animal**

In this study, three sets of ten weeks old female BALB/c mice were orally administered with heroin. For oral administration, 22mg/kg heroin hydrochloride was formulated in saline for injection. Urine samples were collected in 10 ml polypropylene tubes containing 20 mM sodium acetate buffer pH 6.0 to stabilize the amount of heroin. Urine was collected at 0h, 2h, 4h, 6h and 8h respectively, after heroin administration. Urine samples were immediately put on ice after sampling and centrifuged at 2000 g and at 4 °C. Urine was shock frozen in dry ice/methanol and kept at −20 °C until analysis.

**Sample Extraction**

Urine samples (spiked and the samples collected at different time points) were prepared, first mixed with 20 mM sodium acetate buffer pH 6.0 (1:1, v/v) for stabilization of the sample (Klous et al., 2006). The solution was mixed thoroughly, followed by centrifugation at 10000 rpm for 10 min at 4 °C. The supernatant was then collected into fresh sterile tube. This served as the sample for application on TLC plates. The solvent system used for separation of the test samples was chloroform: hexane: triethyl amine (9:9:4). 10 µl sample, with the help of glass capillary, was applied on the TLC plates (10x10 cm). The spots were then dried, cut and dissolve in chloroform: isopropyl alcohol (3:1). The upper aqueous layer was separated and to this chloroform: isopropyl alcohol step was repeated twice. The organic solvent fractions of all the three extraction steps were pooled together. These pooled fractions were then passed through anhydrous sodium sulphate to remove the traces of water. The organic solvent was then evaporated completely and the precipitates were redissolved in 50 µl of methanol and filtered through 0.22 µm filters before being used for further analysis.
Validation methods

[a] MALDI-TOF-MS

The Voyager-DESTR mass spectrometer was run in the ion selective mode. Urine samples (spiked and test samples) were prepared at 0.5 mg/ml in methanol. The screening was performed for all the standards viz. heroin, mono-acetylmorphine, morphine, codeine and test samples at different time intervals, respectively and mixed in equal amounts with sinapinic acid solution (15 mg/ml in acetonitrile), and applied on a stainless steel probe with 0.5 μl of TFA solution (0.1%). The samples were allowed to dry at room temperature and then kept in the system for mass analysis. The data were acquired with 50 shots per sample in the linear mode at 30 kV and analyzed using the software provided with the system. The ions noted in parentheses were used for quantification. The mass ion defect of all quantitative ions (base peak) for standards and tests were determined with standard and urine samples at different time points.

[b] HPLC (high-performance liquid chromatography)

The HPLC system consisted of Shimadzu LC-20AD binary pump (Shimadzu Corporation, Kyoto, Japan). 20 μl sample was injected in an injector (SIL-10 A/10AVP) with a microsyringe (SIL-10ADvp) to a Luna 5μm C18 column (CTO-10AS; 250 mm x 4.60 mm, particle size 5 μm). UV-absorbance detection was taken at 214 nm using UV-vis detector (SPD-10A/10Avp). Gradient elution was performed using a mixture of 5mM ammonium acetate in water (pH 4.0) [A] and acetonitrile [B], for 2 min 95% A and 5% B, 2-8 min 85% A and 15% B, 8-11 min 20% A and 80% B, 11-15 min 95% A and 5% B. Flow rate of the mobile phase was kept constant at 0.5 ml/min and the total run time was 15 min. The column oven temperature was kept at 25 °C. The microsyringe was washed with 100% methanol after each injection.

[c] Competitive ELISA

The conjugate-coated (BSA-MAM - 5 μg/ml) ELISA plates were subjected to competitive immunoassay by adding 100 μl of standard drug solution (heroin, MAM, morphine or codeine) in urine samples and mixing with equal volume of anti-MAM IgG solution (1:500 in PBS). The standard drug solutions were prepared in PBS at
concentrations between 0.01 ng/ml to 10 ng/ml. The plates were incubated for 2 h at 37 °C. After washing, secondary antibody goat anti-rabbit IgG-HRP was added into each well (100 µl/well) and incubated for 1 h at 37 °C. The ELISA plates were washed again and TMB substrate was added to develop the color for 15 min at 37 °C. The reaction was stopped by adding 100 µl 1N H₂SO₄. The absorbance was measured by micropalte ELSIA plate reader (BioTek synergy 2, Finalnd) at 495 nm excitation and 525 nm emission wavelengths.

[d] Competitive Fluoroimmunoassay in spiked and real urine samples

The anti-MAM IgG antibody was labeled with FITC according to the method described earlier (Goding et al, 1976). The conjugate-coated (BSA-MAM-5 µg/ml) ELISA plates were subjected to competitive fluorescence immunoassay by using 100 µl of each standard drug solution (heroin, MAM, morphine or codeine) in spiked urine samples and real urine samples collected at different time points 0, 2, 4, 6, 8, 10 h, in wells and mixing with equal volume of anti-MAM IgG-FITC solution (1:1000 in PBS). The standard drug solutions were prepared in PBS at concentrations between 0.001 ng/ml to 10 ng/ml for spiked urine standard drug samples. The plates were incubated for 2 h at 37 °C. After washing, the fluorescence intensity of each well was measured by fluorescence plate reader (Molecular Devices, USA) at 495 nm excitation and 525 nm emission wavelengths. The %B/Bo for real urine samples were calculated after calibration with standard drug spiked urine samples.

[d] Dipstick based immunoassay

Anti-MAM IgY antibodies and chromatographic dipstick were used for drug detection in urine samples at different time points (0, 2, 4, 6, 8, 10 h). The assay was done as described earlier for dipstick assay for anti-MAM-IgY.
Phage Display of antibodies for MAM detection

Chemicals and reagents

Human single-fold scFv libraries Tomlinson 1+J were obtained from the Medical Research Council (Cambridge, UK) which came with *E. coli* strains TG1 (for phage amplification) (labeled TG1Tr) (K12 Δ(lac-proAB) supE thi hsdD5/F’ traD36 proA+B lacIq lacZΔM15), HB2151 (for scFv production), KM13 helper phage. Tris, skimmed milk powder, Bacto-Agar, Tryptone and Yeast Extract were procured from Hi Media Laboratories. IPTG (isopropyl β-D-thiogalactoside), Ampicillin, Kanamycin, glucose, glycerol, NaCl, Na2HPO4, NaH2PO4·2H2O, PEG 6000, CaCl2, Trypsin T-1426 Type XIII from Bovine Pancreas were procured from Sigma Chemical Company Ltd., USA. Maxisorp immuno test tubes were procured from Tarsons labwares, Chandigarh, India. Nunc Bio-Assay dish, Nunc 24 and 96 well Maxisorp plates were procured from Nunc, Denmark. Horse Radish Peroxidase conjugated Protein A and HRP-anti-M13 were procured from Amersham Biosciences, India. 3, 3′, 5, 5′-Tetramethylbenzidine (TMB) was purchased from Bangalore Genei, India. 0.22 μm filters were purchased from MDI, India. All the experiments were done according to the guidelines provided by the manufacturer's instructions.

Production of large quantities of helper phage (KM13)

For large scale production of helper phage, take 200 μl TG1 cells (OD600 = 0.4) with 10 μl of KM13 helper phage and incubated at 37 °C for 30 min in water bath (without shaking). Take 100 μl TG1 and added into 3 ml molten H-top agar (pre-warmed at 42 °C) and poured onto TYE plates (no antibiotics). Allowed to set and incubated overnight at 37 °C. Pick a small plaque into 5 ml of fresh TG1 (OD600 = 0.4). The culture was grown for 2 hr at 37 °C and transferred to 500 ml 2xTY and grown at 37 °C for 1 hr. 50 μg/ml kanamycin was added and grown overnight at 30 °C. Centrifuge the overnight grown culture at 10,800 x g for 15 min. 100 ml PEG/NaCl (20% polyethylene glycol 6000, 2.5 M NaCl) was added to 400 ml supernatant and leave for 1 hr on ice and centrifuged at 10,800 g for 30 min. Resuspended the pellet in 8 ml PBS and added 2 ml of PEG/NaCl and leave for
20 min on ice. Centrifuge at 3300 g for 30 min and resuspended the pellet in 5 ml PBS and spin at 11,600 g for 10 min. Stored the helper phage at 4 °C for short term storage or in PBS with 15 % glycerol for longer term storage at -70 °C.

To titre the helper phage, take 45 μl phage and add 5 μl trypsin stock solution and incubated for 30 min at 37 °C. Take 1μl of trypsin treated phage in 1ml of PBS and prepare five 100 fold serial dilutions in 1ml of PBS. 50 μl of the six dilutions were transferred to six separate tubes containing 1ml of TG1 (OD$_{600}$ = 0.4). 3 ml of molten H-Top agar was added and poured onto TYE plates. Same dilution series was used for 1 μl of non-trypsin treated phage.

**Growing the libraries (I+J)**

Add 500 μl library stock (I+J) separately to 200 ml 2 x TY (100 μg/ml ampicillin and 1 % glucose) and incubated at 37 °C until the OD$_{600}$ was 0.4. Take 50 ml of this and 2 x10$^{11}$ KM13 helper phage was added. (Use the remaining 150 ml to make a secondary bacterial stock of the library) and incubated at 37 °C for 30 min. Centrifuge at 3,000 g for 10 min and resuspended in 100 ml 2 x TY (100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1% glucose) and incubated at 30 °C overnight followed by centrifugation at 3300 g for 30 min. 20 ml PEG/NaCl was added into 80 ml supernatant and leave for 1 h on ice followed by centrifugation at 3300 g for 30 min. Resuspended the pellet in 4 ml PBS and centrifuged at 11600 g for 10 min and stored the phage at 4 °C or 15% glycerol and stored at -70 °C.

To titre the phage stock, take 1μl phage in 100 μl PBS, 1μl of this in 100 μl PBS and so on until 6 dilutions in total. Take 900 μl of TG1 (OD$_{600}$ = 0.4) to each dilution and incubate at 37 °C for 30 min. Spread 100 μl of each dilution on a TYE plate (100 μg/ml ampicillin + 1% glucose) and incubated overnight at 37 °C. Phage stock should be 10$^{12}$-10$^{13}$/ml, enough for at least 10 selections.

**Growing secondary stocks of the libraries**

Grow the remaining 150 ml (I+J) for further 2 h at 37 °C followed by centrifugation at 10,800 g for 10 min and resuspended in 10 ml 2 x TY containing 15 % glycerol. Stored this secondary stock in 20 x 500 μl aliquots at -70°C.
Panning of MAM-specific binding clones from the phage antibody library

Coat the immunotubes (40 μg/ml) with BSA-MAM conjugate and kept overnight at 4°C. Washed three times with PBS and blocking was done for 2 h at RT with 2% skimmed milk (in PBS) followed by washing the tube three times with PBS. The helper phage was added at 10^13 phage into 4 ml of 2% PBSM and incubated for 1 h at RT, rotating and then stand for a further 1 h at RT. Wash tubes 10 (1st round of panning) and later with 20 times in subsequent panning with PBS containing 0.1% Tween 20. Eluted the phage by adding 500 μl of trypsin-PBS (50 μl of 10 mg/ml trypsin stock solution + 450 μl PBS) and rotating for 10 min at RT. Take 1.75 ml TG1 (OD₆₀₀ = 0.4) and added 250 μl of the eluted phage and incubated for 30 min at 37 °C in a water bath without shaking. Spread with the help of spreader 100 μl, 100 μl of a 1:10² dilution and 10 μl of a 1: 10⁴ dilution on TYE plates (100 μg/ml ampicillin + 1 % glucose) and was grown overnight at 37 °C. Take the remaining TG1 culture and centrifuge at 11600 g for 5 min. Pellet of bacteria was resuspended in 50 μl of 2xTY and plate on TYE plate (100 μg/ml ampicillin + 1 % glucose). Grow plates at 37 °C overnight.

Further rounds of selection

After overnight growth, added 2 ml to the TYE plate and loosen the cells with a glass spreader. Inoculate 50 μl of the scraped bacteria to 50 ml of 2xTY (100 μg/ml ampicillin + 1% glucose) and stored 1 ml of the remaining bacteria at -70 °C in 15% glycerol. Incubated at 37 °C until OD₆₀₀ = 0.4. To 10 ml of this culture, added 5 x 10¹⁰ helper phage and incubated at 37 °C in water bath for 30 min. Spin at 3,000 g for 10 min and resuspended in 50 ml of 2 x TY (100 μg/ml ampicillin, 50 μg/ml kanamycin and 0.1% glucose). Incubated at 30 °C overnight and centrifuge the overnight culture at 3300 g for 15 min. 10 ml PEG/NaCl was added to 40 ml supernatant and leave for 1 hr on ice followed by centrifugation at 3300 g for 30 min. The pellet was resuspended in 2 ml PBS and spins at 11600 g for 10 min. Use 1 ml of this phage for the next round of selection and stored 1 ml at 4 °C. Ten rounds of panning were carried out in total. To reduce the number of false positive phage clones, the following modifications to the protocol was done. First, we added a pre-absorption step in first three rounds of selection with BSA conjugate. The phage solution was incubated with a coated tube for 30 min then
transferred to another coated tube. In the fourth round of selection, immunotubes were coated with BSA-MAM conjugate for specific phage collection.

**Screening of MAM specific clones by polyclonal phage ELISA**

Populations of phage produced at each round of selection can be screened for binding by ELISA to identify "polyclonal" phage antibodies. Phage from single colonies can then be screened by ELISA to identify "monoclonal" phage antibodies. Alternatively, after a polyclonal phage ELISA we could proceed directly to making monoclonal soluble antibody fragments.

96 well ELISA plates were coated (40 µg/ml) 100 µl/well of BSA-MAM in 50 mM carbonate buffer and incubated overnight at 4 °C. Washing was done three times with PBS. Blocking was done with 2% PBSM (200 µl/well) and incubated for 2 h at RT. Washing was done as before. 10 µl PEG precipitated phage from the end of each round of selection was added into 100 µl of 2% PBSM and incubated for 1 h at RT. Phage solution was discarded and the plates were washed three times with PBST. Added 1:3000 dilution of HRP-anti-M13 in 2% PBSM and incubated for 1 h at RT followed by washing three times with PBST. 100 µl/well TMB substrate was added and incubated at RT for 15 min. Blue colour was developed. Stop the reaction by adding 50 µl 1 N sulphuric acid. The blue color was turned into yellow. Read the OD₄₅₀ nm.

**Production of soluble antibody fragments**

From each selection round, 10 µl of eluted phage was taken and infected 200 µl exponentially growing HB2151 bacteria (OD₆₀₀ = 0.4) and incubated for 30 min at 37°C. 50 µl, 50 µl of a 1:10² dilution, 50 µl of a 1:10⁴ dilution and 50 µl of a 1:10⁶ dilution was plated onto TYE plates (100 µg/ml ampicillin + 1% glucose) and incubated overnight at 37 °C. Pick individual colonies into 100 µl 2xTY (100 µg/ml ampicillin + 1% glucose) in 24 well plates and incubated overnight at 37 °C. Transferred 2 µl inoculum from this plate to a second 24 well plate 200 µl 2xTY (100 µg/ml ampicillin + 0.1% glucose/well) and incubated at 37°C until OD₆₀₀ = 0.9. Once OD₆₀₀ = 0.9 was reached then added 25 µl 2xTY (100 µg/ml ampicillin + 9 mM IPTG, final concentration 1 mM) and incubated at 30 °C overnight. Coat a 96 well flexible assay plate overnight with 100 µl/well of MAM-BSA (40
μg/ml) in 50mM carbonate buffer. Spin the overnight grown ELISA plate at 1800 g for 10 min and use 100 μl of the supernatant for monoclonal phage ELISA.

**Screening and selection of MAM positive clones**

For this, 96 well ELISA plates were coated (40 μg/ml) 100 μl/well of BSA-MAM in 50 mM carbonate buffer and incubated overnight at 4 °C. Washing was done three times with PBS. Blocking was done with 2% PBSM (200 μl/well) and incubated for 1 h at RT. Washing was done as before. 100 μl Individual colonies (supernatant) were added from the titration plates into 96 well ELISA plates and incubated for 2 h at RT. Phage solution was discarded and the plates were washed three times with PBST. Added 1:3000 dilution of Protein L-HRP in 2% PBSM and incubated for 1 h at RT followed by washing three times with PBST. 100 μl/well (1x) TMB substrate was added and incubated at RT for 20 min. Blue colour was developed. Stop the reaction by adding 50 μl of 1 M sulphuric acid. The blue color was turned into yellow. Absorbance was measured at OD₄₅₀ nm.

**Characterization of MAM positive clones**

[a] **Expression of soluble scFvs**

[i] **SDS-PAGE**

All MAM positive scFv antibodies separation was run under reduced conditions on 12% polyacrylamide gels with a 4% stacking gel according to the method of Laemmli (1970). Clarified periplasmic extract was obtained by centrifugation of the resuspended product at 12000 rpm for 10 min. Protein estimation was done by Bradford method (Lowry et al., 1951). Then, the periplasmic extract was mixed with 5μl of 2 × SDS-sample buffer and loaded into wells for SDS-PAGE analysis.

[ii] **Western blot**

All seven selected MAM positive scFv clones positive for MAM, along with control was run in each lane of SDS-PAGE as described above and transferred to nitrocellulose membrane. The membrane was then blocked with 5% skimmed milk in PBS, pH 7.4.
ImmunoPure protein L-HRP (Pierce) (1:1000) was used to detect bound scFvs in PBST at 4°C overnight. TMB substrate was added to a final concentration of 1x to develop a colour and finally stop further colour development by placing the strips in DW (According to the guidelines provided by Pierce).

[b] Plasmid isolation and DNA sequencing of scFv clones

Plasmid DNA from all the seven clones were isolated by QIagen miniprep plasmid isolation kit (QIAgen, Hamburg, Germany) and 2 μl of plasmid DNA were analyzed on 1% (w/v) agarose gel in 1x TAE to check the purity and concentration of the plasmid DNA, which was further confirmed by Nanodrop (NanoVue Plus Spectrophotometer, Amershan Biosciences, India). Primers used for DNA sequencing were LMB3 (forward primer) (CAG GAA AC A GCT ATG AC) and pHEN (reverse primer) (CTA TGC GGC CCC ATT CA). Sequencing was done by ABI Big Dye v3.1 Cycle Sequencing protocol by automated sequencing. PCR reactions were carried out in 10 μl reaction mixture containing 1.0 μl Terminator Ready Reaction Mix, 5 x Sequencing Buffer 1.5 μl, Template 200 ng, each primer 3.2 pmol. PCR consisted of 24 cycles of denaturation at 96 °C for 1 min, annealing at 50 °C for 5 sec and polymerisation at 60 °C for 4 min. Post reaction clean up and resuspension was done to remove the unincorporated dye terminators from the sequencing reaction. Briefly, 2 μl of 125 mM EDTA was added to the PCR product and 50 μl of 95 % ethanol were added to the each tube followed by incubation at RT for 15 min. The PCR tubes were then centrifuged at 12000 rpm for 20 min at room temperature and 250 μl of 70% ethanol were added and repeat the centrifugation for 10 min. Repeat the was again and air dry the pellet for 45 min and 10 μl of HiDi Formamide was added to the dried pellet followed by heating at 95°C for 3 min. Immediately kept on ice for 5 min and sequencing was done by dideoxy method.

[c] Reactivity of scFv MAM antibodies

ELISA plates were coated with 100 μl of BSA-MAM conjugate (40 μg/ml) prepared in carbonate buffer (20 mM, pH 9.6) and incubated overnight at 4 °C. The plates were subsequently washed thoroughly with PBS (50 mM, pH 7.4). Unbound sites were
blocked with 2% defatted skimmed milk in PBS for 1 h at 37 °C. The plates were then washed thrice with PBS containing 0.05% Tween-20 (PBST) and once with PBS. All soluble antibody dilutions were made in sterile filtered PBS, 1% (w/v) non-fat milk powder at a volume of 100 µl/well and incubations were preformed at 37 °C for 1 h. Decreasing concentrations of scFv were added to each well. After washing, 100 µl of Protein-L-HRP (1:3000 dilution in PBSM) was added into each well and incubated for 1 h at 37 °C. Finally, 100 µl of substrate (TMB) was added into each well. The enzymatic reaction was carried out in dark for 25 min at RT. The reaction was stopped by adding 50 µl of 1N H₂SO₄ into each well, followed by measurement of absorbance at 450 nm using the ELISA plate reader (Biotek- XS Plus).

[d] Competitive ELISA
Competitive studies were performed in the same manner as for standard ELISA except decreasing concentrations of free MAM and other opiate drugs (viz. heroin, morphine and codeine respectively) (50 µl) were incubated with scFv (50 µl) on the microtitre plate for 2 h at 37 °C. Protein L-HRP was used as secondary antibody for the detection of soluble scFv.

e] BIAcore-based immunoassay

[i] Coupling reaction of MAM-BSA to CM5 dextran gel
The carboxymethylated dextran (CM-dextran) matrix was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride) prepared in ultra-pure water and injecting the mixture over the sensor chip surface for 7 min at a flow rate of 5 µl/min. The BSA-MAM conjugate to be immobilized (concentration, 100 µg/ml) was dissolved in 10 mM sodium acetate buffer, pH 4.5, and injected over the surface for 40 min at a flow rate of 5 µl/min. The unreacted sites on the sensor chip surface were then capped by injection of 1M ethanolamine prepared in ultra-pure water, pH 8.5, for 7 min (Brennan et al., 2003).
Sample preparation for sensor analysis and regeneration conditions required

Seven different MAM positive scFv antibodies were diluted in HBS buffered saline solution (HBS, pH 7.4) at a concentration 100 µg/ml, separately. All buffers and solutions used were made up using ultrapure water, degassed and sterile filtered. Regeneration conditions for the removal of the scFv antibody from the surface of the chip were optimized and found to require a 1 min pulse of 10 mM Glycine-HCl, pH 3.0.