BILATERAL SCIENTIFIC AND TECHNOLOGICAL COOPERATION BETWEEN THE REPUBLIC OF INDIA AND THE RUSSIAN FEDERATION IN THE AREA OF BIOTECHNOLOGY

DEPARTMENT OF BIOTECHNOLOGY

MINISTRY OF SCIENCE AND TECHNOLOGY, GOVT. OF INDIA

&

RMES, RUSSIA

FUNDED RESEARCH PROJECT

Application of snake venom toxins labeled with functionalized nanoparticles for detecting endogenous targets in cells and ex vivo tissues with prospects for the development of novel diagnostic and therapy tools.

FINAL REPORT

(19th September, 2014 to 18th September, 2017)

Submitted By

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RMES, RUSSIA

Proforma for Joint Evaluation of Final Progress Report submitted for a project against Joint Call for Proposal (2014-16)

1	**Duration of the report:**	19th September, 2014 to 18th September, 2017
2	**Project Title**	Application of snake venom toxins labeled with functionalized nanoparticles for detecting endogenous targets in cells and ex vivo tissues with prospects for the development of novel diagnostic and therapy tools.
3	**Principle Investigators:**	
	i. Indian	Dr. Ashis Kumar Mukherjee, Tezpur University, Tezpur
	ii. Russian	Dr. Yuri Utkin, National University of Science and Technology, MISIS, Moscow
4	**Objectives sanctioned**	**1**. Isolation, purification and characterization (*in vitro* activity study) of alpha-neurotoxins, nerve growth factor from venom samples of cobra (*Naja naja* and/or *Naja kaouthia*) and krait (*Bungarus caeruleus* and/or *Bungarus fasciatus)* and nerve growth factor from viper venoms (Vipera species). **2**. To prepare the above mentioned toxins labelled with functional nanoparticles as reporter groups. **3**. Investigation of interaction of nanoparticles conjugated toxins with receptors (the receptor-ligand binding study) at the level of cells and tissue of normal as well as of malignant origin.

5	**Objectives achieved (Indian Partner)**	**1.** Isolation, purification, and characterization of biological activity of alpha-neurotoxins and nerve growth factor (NGF) from venom samples of cobra (*Naja naja, N. kaouthia*) and Russell's viper (*Daboia russelii*). **2.** Assessment of *in vitro* cytotoxicity of above purified proteins against normal (PC-12) and malignant (breast cancer MCF-7 and MDA-MB-231) cells and haemolytic activity against mammalian erythrocytes. **3.** Investigation of interaction of above purified proteins with cell surface TrkA receptor (the receptor-ligand binding study) highly expressed in normal (PC-12) and malignant (breast cancer MCF-7 cells).
	Objectives achieved (Russian Partner)	**1.** Synthesis of fluorescence CdSe quantum dots and llabelling/conjugation of purified alpha-neurotoxins and NGF with functional nanoparticles as reporter groups. **2.** Assessment of cytotoxicity of functionalized fluorescence nanoparticles conjugated with NGFs, short neurotoxin 1, and alpha-elapitoxin-Nk2a against breast cancer cells. **3.** Investigation of interaction of nanoparticles/quantum dots conjugated NGF and alpha-neurotoxins with cell surface receptors (the receptor-ligand binding study).
6	**Human resource development (Indian Partner)**	
	i. JRF/SRF	The JRF appointed in this project is registered for Ph.D. degree at Tezpur University.

	ii. **PostDoc/RA**	No provision for Potdoc/RA

7 **Research Outcome**

i. Publication (list may be included as annexure)	Yes (2 publication in peer reviewed journal, 1 under preparation)
ii. Patents	No
iii. Technology developed / commercialized	Yes but not commercialized

8 **Summary of progress work: (Indian partner)**

Nerve growth factor (NGF) isoforms and alpha-neurotoxins were isolated and purified from Indian Russell's Viper (*Daboia russelii*), and cobra (*N. naja* and *N. kaouthia*) venom samples. The molecular mass and purity of above proteins were confirmed by SDS-PAGE and/or mass spectrometry analyses and they were identified by tandem mass spectrometry (LC-MS/MS) analysis. The nerve growth factors were also characterized for their biological activity to induce neurite outgrowth in PC-12 neuronal cells. The cytotoxicity of the above proteins against mammalian normal PC-12 (neuronal) cells as well as malignant breast cancer cells (MCF-7 and MDA-MB-231) were assayed in *in vitro* and they were found to be devoid of cytotoxicity. Further, the above proteins/toxins did not induce hemolysis of the mammalian erythrocytes. The binding of NGF and alpha-neurotoxins to cell surface Trk group of receptors highly expressed in PC-12 (normal neuronal cell

line) and breast cancer MCF-7 cells assayed by ELISA and Immuno fluorescence analyses demonstrated that these toxins bind very specifically to TrkA receptor subtype of the above cells. This study indicates that the snake venom proteins under study are very promising candidates for the development of diagnostic reagent to detect breast cancer of other cancer overexpressing TrkA receptor on cell surface.

Summary of progress work: (Russian partner)

Fluorescent CdSe quantum dots/ nanoparticles with different spectral properties were synthesized. Their surface was functionalized with thiol-containing compounds and the conjugates of alpha-cobratoxin and nerve growth factors with fluorescent nanoparticles were prepared. Influence of fluorescent nanoparticles functionalized with glutathione on the survival of the PC-12 and MCF-7 cell lines was assessed and no cytotoxicity was detected. The cytotoxicity of alpha-cobrotoxin conjugated with fluorescent nanoparticles was also determined against MCF-7 and PC-12 cells and the toxin-fluorescent Nano conjugates were found to be non-toxic. The conjugates of alpha-cobrotoxins and NGF with fluorescent CdSe quantum dots were used for visualization of TrkA and α7 nicotinic acetylcholine receptor. Because quantum dots produce highly bright fluorescent signal and resistance to bleaching therefore, our study suggest that CdSe labelled with snake venom NGF or alpha-cobrotoxin are potent candidate for development of diagnostic reagent for cancer.

9. Brief progress report (Indian Partner)

A. Isolation, purification, and characterization of biological activity of alpha-neurotoxins and nerve growth factors (NGFs) from venom samples of cobra (*Naja naja / N. kaouthia*) and Russell's viper (*Daboia russelii*).

A1. Isolation, purification, and identification of nerve growth factor from Indian Russell's Viper (*Daboia russelii*) venom

Fractionation of *D. russelii* venom (200 mg) dissolved in 1.0 ml of 25 mM HEPES buffer containing 50 mM NaCl (pH 7.0) on a HiLoad 16/600 Superdex 75 pg FPLC gel-filtration column resulted in separation of venom proteins to 11 fractions (GF1 to GF-11). The desalted gel-filtration peak RVVGF3 showed neuritogenesis potency in PC-12 cells derived from pheochromocytoma of the rat adrenal medulla that has an embryonic origin from the neural crest to detect the presence of neurotropin-like molecule (assay conditions are mentioned below). This fraction was further separated in FPLC anion-exchange HiQ column and proteins eluted from this column with 0-500 mM linear gradient of NaCl were separated in 17 peaks. Out of the 17 anion-exchange peaks, the proteins of two peaks GF3HiQ5 and GF3HiQ6 eluted at NaCl concentration of 100-120 mM showed neuritogenesis activity in PC-12 cells indicating they contain NGF.

The FPLC anion-exchange peaks GF3HiQ5 and GF3HiQ6 were re-fractionated in RP-UHPLC C_{18} (Acclaim 300, 4.6 x150mm, 3 µm) column. The RP-UHPLC peaks (Figs. 1A, B) showed neuritogenesis activity in PC-12 cells suggesting these are NGF isoforms from Russell's viper venom.

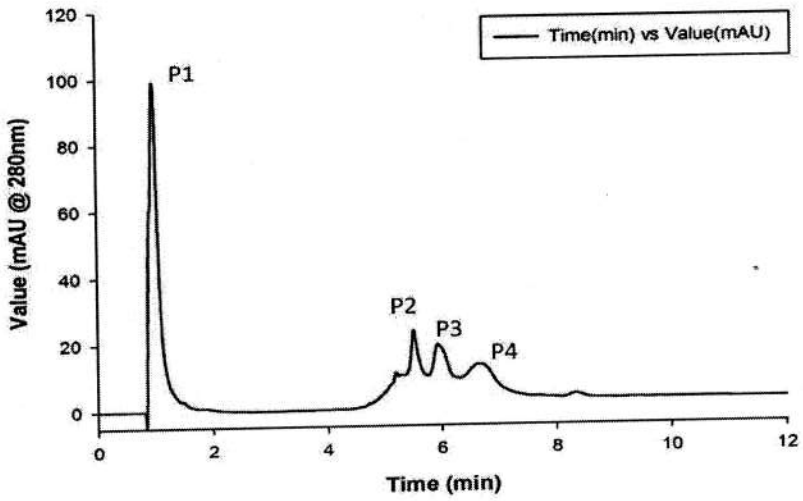


Fig. 1A. RP-UHPLC purification of NGF from RVV(Hi) GF3 HiQ 5 fraction. Flow rate was 30 ml /min and elution of protein was monitored at 280 nm. Fraction volume was 0.25 ml.

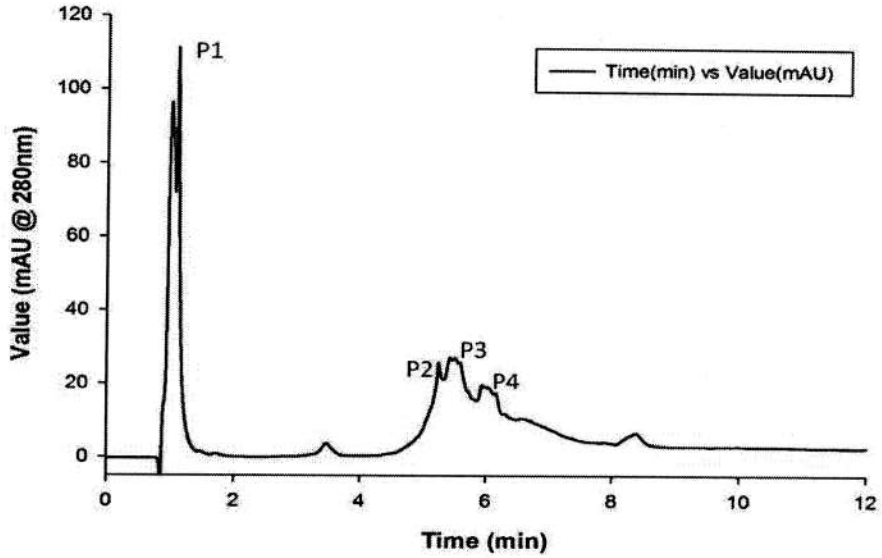


Fig. 1b. RP-UHPLC purification of NGF from RVV(Hi) GF3 HiQ 6 fraction. Flow rate was 30 ml /min and elution of protein was monitored at 280 nm. Fraction volume was 0.25 ml.

The purity and molecular mass of Russell's viper NGFs were determined by MALDI-ToF-MS analysis and their mass was determined at m/z 27.4, and 13.4 which are in the molecular mass range of snake venom NGFs.

The identity of the purified NGFs was further confirmed by LC-MS/MS analysis of tryptic digested proteins (Dutta, Chanda et al. 2017, Kalita, Patra et al. 2017). The generated data was searched for their identity using MASCOT 2.4 search engine against the Uniprot Swiss-Prot database (non-redundant database with reviewed proteins) against viperidae venom from NCBI and the data were analyzed on Proteome Discoverer 1.4 software (Mukherjee, Dutta et al. 2016, Kalita, Patra et al. 2017). The proteomic analysis showed the identity of the protein eluted in RP-HPLC peak 1 with NGF sequences from snake venom (Table 1). The alignment of their sequences is shown in Fig. 2.

Table 1. Identification of purified NGFs from Russell's viper (*D. russelii*) venom by LC-MS/MS analysis of RVVGF3HiQ5P1 and RVVGF3HiQ6P1 fractions.

RVVGF3HiQ5P1						
Accession	**Description**	**Score**	**Coverage**	**MS-MS derived sequences**	**MW [kDa]**	**Cal PI**
400499	Venom nerve growth factor; Short=v-NGF; Short=vNGF	1233.21	38.46	INTAcVcVISR HWNSYcTTTDTFVR cKNPNPVPSGcR cKNPNPVPSGcR INTAcVcVISRK qYFFETK	13.3	8.50
335892642	venom nerve growth factor 2 [Daboia russelii]	1233.21	18.52	INTAcVcVISR HWNSYcTTTDTFVR cKNPNPVPSGcR cKNPNPVPSGcR INTAcVcVISRK qYFFETK	27.4	8.62
335892640	venom nerve growth factor 1 [Daboia russelii]	1233.21	18.52	INTAcVcVISR HWNSYcTTTDTFVR cKNPNPVPSGcR cKNPNPVPSGcR INTAcVcVISRK qYFFETK	27.4	8.46

RVVGF3HiQ6P1						
Accession	**Description**	**Score**	**Coverage**	**MS-MS derived sequences**	**MW [kDa]**	**Cal PI**
400499	RecName: Full=Venom nerve growth factor; Short=v-NGF; Short=vNGF	1147.09	31.62	INTAcVcVISR HWNSYcTTTDTFVR cKNPNPVPSGcR cKNPNPVPSGcR	13.3	8.50
335892642	venom nerve growth factor 2 [*Daboia russelii*]	1147.09	15.23	INTAcVcVISR HWNSYcTTTDTFVR cKNPNPVPSGcR cKNPNPVPSGcR INTAcVcVISRK qYFFETK	27.4	8.62
335892640	venom nerve growth factor 1 [*Daboia russelii*]	1147.09	15.23	INTAcVcVISR HWNSYcTTTDTFVR cKNPNPVPSGcR cKNPNPVPSGcR INTAcVcVISRK qYFFETK	27.4	8.46

Fig.2. Alignment of MS-MS derived tryptic peptide sequences with the identified NGF from RVV. The sequences shown in color are tryptic peptide sequences.

- **Accession No: 400499 [Venom nerve growth factor from *Daboia russelii russelii*]**

HPVHNQGEFSVCDSVSVWVANKTTATDMRGNVVTVMVDVNLNNNVYK**QYFFETKCKNPNPVPSGCR**GIDA
K**HWNSYCTTTDTFVR**ALTMERNQASWRF**IRINTACVCVISR**KNDNFG

- **Accession No: 335892642 [Venom nerve growth factor 2 from *Daboia russelii russelii*]**

MSMLCYTLIIAFLIGIRAAPKSEDNVSLRSPATPDLSDTSCAKTHEALKTSRNTDQHYPAPNKAEDQEFG
SAANIIVDPKLFQKRRFQSPRVLFSTQPPPLSRDEQSVEFLDNADSLNRNIRAKRATHPVHNQGEFSVCD
SVSVWVANKTTATDMRGNVVTVMVDVNLNNNVYK**QYFFETKCKNPNPVPSGCR**GIDAK**HWNSYCTTTDTF**
VRALTMERNQASWRFIR**INTACVCVISR**KNDNF

- **Accession No: 335892640 [Venom nerve growth factor 1 from *Daboia russelii*]**

MSMLCYTLIIAFLIGIWAAPKSEDNVSLRSPATPDLSDNSCAKTHEALKTSRNTDQHYPAPNKAEDQEFG
SAANIIVDPKLFQKRRFQSPRVLFSTQPPPLSRDEQSVEFLDNADSLNRNIRAKRATHPVHNQGEFSVCD
SVSVWVANKTTATDMRGNVVTVMVDVNLNNNVYK**QYFFETKCKNPNPVPSGCR**GIDAK**HWNSYCTTTDTF**
VRALTMERNQASWRFIR**INTACVCVISR**KNDNF

A2. Confirmation of biological activity (neurite outgrowth-inducing property) of NGF purified from *D. russelii* venom on PC-12 cells

The neurite outgrowth-inducing property of two RP-HPLC purified proteins viz. RVVGF3HiQ5P1 and RVVGF3HiQ6P1 from *D. r. russelii* venom (demonstrated sequence homology with NGF) was assayed against PC-12 cells obtained from American Type Culture Collection (ATCC, USA). The cells at a cell density of 5 x 10^4 cells per well were grown in 6 well cell culture plate in DMEM in presence or absence of 10% FBS, at 37 °C, 5% CO_2 for 14 days. The culture medium was replaced at an interval of 3 days. The culture conditions were standardized using commercial Nerve Growth Factor (Sigma Aldrich, Missouri, USA) derived from mouse submaxillary glands. The cells showed distinct neurite outgrowth for the exogenously added mouse NGF/RVV-NGF at concentrations of 100 ng/ml. (Fig. 3). This bioassay confirmed that the purified proteins are NGF isoforms from Russell's viper (*D. r. russelii*) venom. This is the first report on purification of NGF isoforms from Indian Russell's Viper venom.

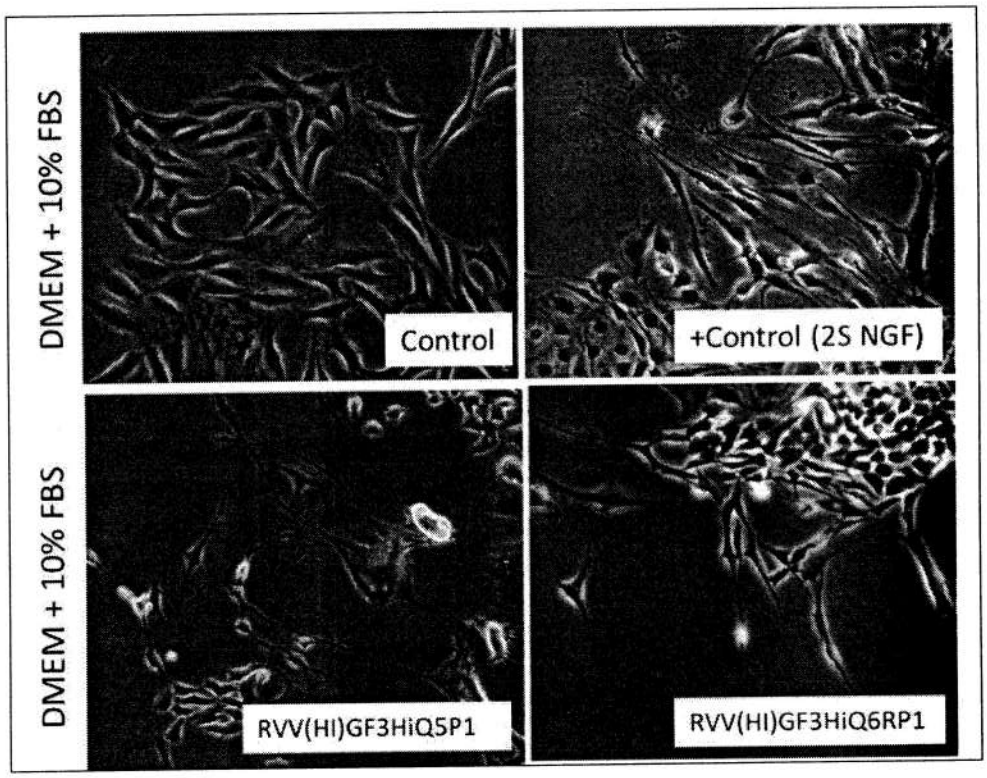


Fig 3. PC-12 cells grown in DMEM containing 10% FBS showing positive neurite growth in presence of NGFs purified from Russell's viper venom.

A3. Isolation, purification, and identification of alpha-neurotoxins from cobra (*N. naja* / *N. kaouthia*) venom

Fractionation of crude *N. naja* venom (25 mg) on an FPLC HiPrep CM FF 16/10 cation-exchange column resulted in separation of venom proteins into 11 fractions [Nn(N)CEX P1-P11]. The LC-MS/MS analysis of *N. naja* venom peaks suggested that short chain and long-chain α-neurotoxins and cobrotoxins are predominated in peaks 5 to 9 whereas peaks 9 and 10 contained cardiotoxins and cytotoxins. NGF was found to present in peaks 7 and 8.

The desalted pooled fractions 7 and 8 were subjected to reversed-phase ultrahigh performance chromatography (RP-UHPLC) fractionation on a Acclaim 300, C_{18}, 4.6 x150 mm,3.0 µm column equilibrated with the solvent A (100% MilliQ containing 0.1% TFA). After washing the unbound proteins with solvent A, bound proteins were eluted by multisteps gradient of solvent B(90% ACN containing 0.1% TFA). The flow rate was adjusted to 0.5 ml/min, fraction volume was 500 µl and elution of proteins/peptides was monitored at 280 nm (Figs. 4 A,B).

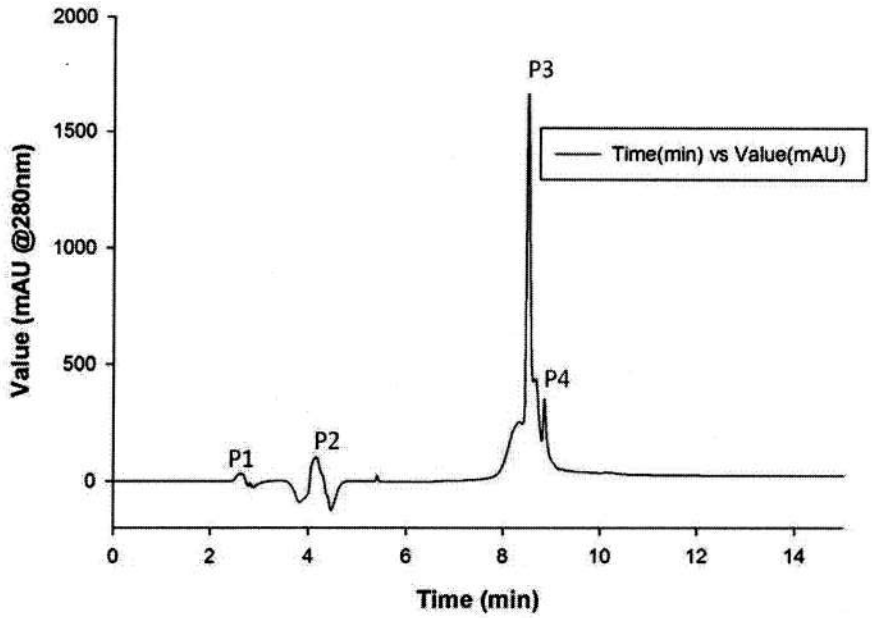


Fig 4A: RP-UHPLC fractionation of Nn(N)CEX P7 fraction.

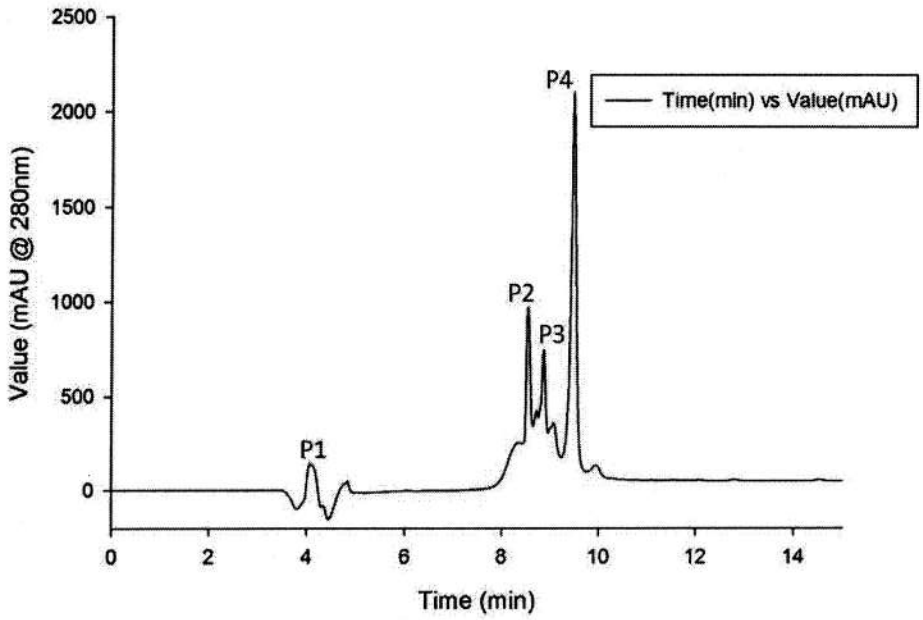


Fig 4B. RP-UHPLC fractionation of Nk(N)CEX P8 fraction.

The molecular mass and purity of protein in each RP-HPLC peak were determined by MALDI-ToF-MS analysis. Their masses were determined at m/z 6925 for Short neurotoxin 1 and 7883 for Alpha-elapitoxin-Nk2a.

The tryptic digested RP-UHPLC purified proteins were subjected to LC-MS/MS analysis to identify the proteins by following the procedures as described by us (Dutta, Chanda et al. 2017). The MS/MS data were searched using MASCOT 2.4 search engine against the Uniprot Swiss-Prot database (non-redundant database with reviewed proteins) against Elapidae venom from NCBI and the data were analyzed on Proteome Discoverer 1.4 software(Mukherjee, Dutta et al. 2016, Kalita, Patra et al. 2017). The proteomic analysis showed the identity of the protein eluted in RP-HPLC peak with alpha-neurotoxin and short neurotoxin sequences from cobra venom (Table 2).

Table 2. LC-MS/MS identification of alpha-neurotoxins purified from *N. naja* / *N. kaouthia* venom.

RP-UHPLC peak no.	**Protein recovered (µg)**	**MS-MS derived peptide sequences**	**Identified protein/peptide**	**Snake Species**
Nn(N)CEX P7RP1	5.4		Insufficient protein for PMF	-
Nn(N)CEX P7RP2	40.5		Could not be identified	-
Nn(N)CEX P7RP3	33.9	LECHNQQSSQAPTTK TCSGETNCYKTCSGETNCYK LECHNQQSSQAPTTK	Short neurotoxin 1	*Naja philippinensis*
Nn(N)CEX P7RP4	36.9	LECHNQQSSQAPTTK TCSGETNCYK LECHNQQSSQAPTTK	Short neurotoxin 1	*Naja philippinensis*
		CFITPDITSK	Alpha-elapitoxin-Nk2a	*Naja kaouthia*
Nk(N)CEX P8RP1	18.5	VKPGVNLNCCR LECHNQQSSQAPTTK TCSGETNCYK	Cobrotoxin-c	*Naja kaouthia*

		VKPGVNLNCCR LECHNQQSSQAPTTK		
		LECHDQQSSQTPTTTGCSGGETNCYK	Neurotoxin 3	*Naja sputatrix*
Nk(N)CEX P8RP2	41.4	No significant hit	Unidentified*	
Nk(N)CEX P8RP3	14.05	CFITPDITSK	Alpha-elapitoxin-Nk2a	*Naja kaouthia*
Nk(N)CEX P8RP4	71.77	LECHNQQSSQAPTTK TCSGETNCYK TCSGETNCYK LECHNQQSSQAPTTK LECHNQQSSQAPTTK	Short neurotoxin 1	*Naja philippinensis*

B. Assessment of *in vitro* cytotoxicity of NGF and alpha-cobrotoxins/ long chain neurotoxins against normal (PC-12) and malignant (breast cancer MCF-7 and MDA-MB-231) cells and haemolytic activity against mammalian erythrocytes

The *in vitro* cytotoxicity assay of NGFs against the breast cancer cells MCF-7 and MDA-MB-231 and normal PC-12 cells was done as described previously (Mukherjee and Mackessy 2013, Mukherjee, Saviola et al. 2015). The purified RVV NGFs at a concentration of 200 ng/ml did not show cytotoxicity against the normal as well as malignant cells (Fig. 5A) suggesting they are devoid of cytotoxicity and may find diagnostic application for *in vitro* and endogenous detection of cancer.

Further, the RVV NGF isoforms at a concentration of 1 mg/ml did not show hemolytic activity against washed mammalian erythrocytes.

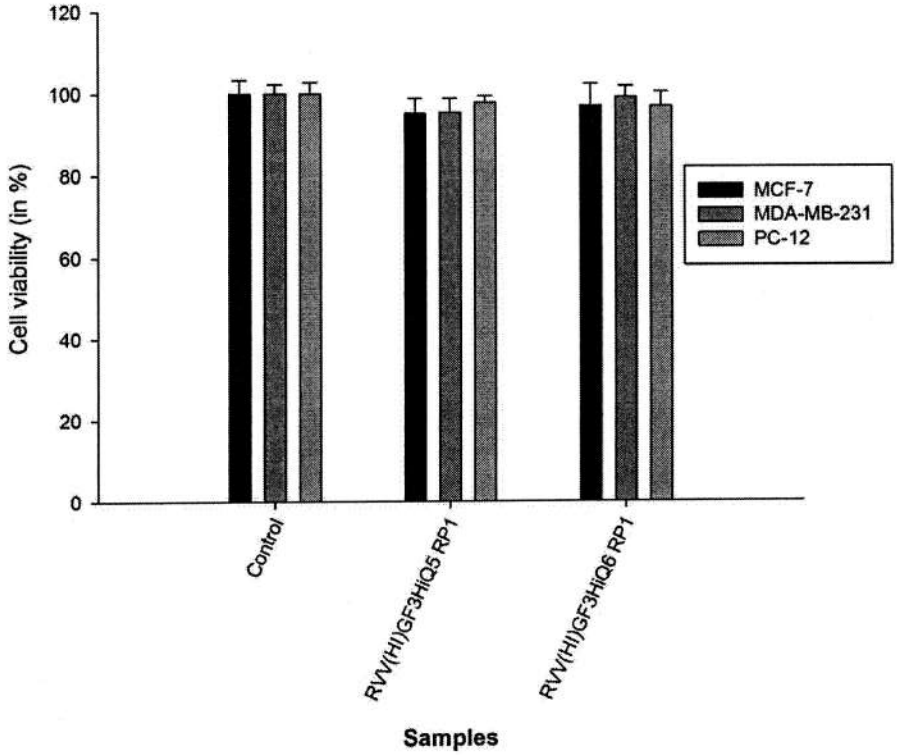


Fig 5A: Cell cytotoxicity of RVV NGFs (200 nM) against PC-12 and human breast cancer cells. Values are mean ±SD of triplicate determinations. The survival of treated cells did not differ from the control cells ($p>0.05$).

The *in vitro* cytotoxicity of purified short neurotoxin 1 and Alpha-elapitoxin-Nk2a at two different concentrations (100 and 200 ng/ml) were assessed against MCF-7, MDA-MB-231, and PC-12 cells by MTT-based method (Mukherjee, Saviola et al. 2015). They were also devoid of cytotoxicity against these cells (Figs. 5B, C). It is to be noted that snake venom toxins will not show cytotoxicity against every cell type but their activity will limited to target cells only. Therefore, the cells under study are not target of the RVV NGF or cobra venom neurotoxins.

As expected the cobra venom neurotoxins at a concentration of 1 mg/ml did not show appreciable hemolytic activity against washed erythrocytes.

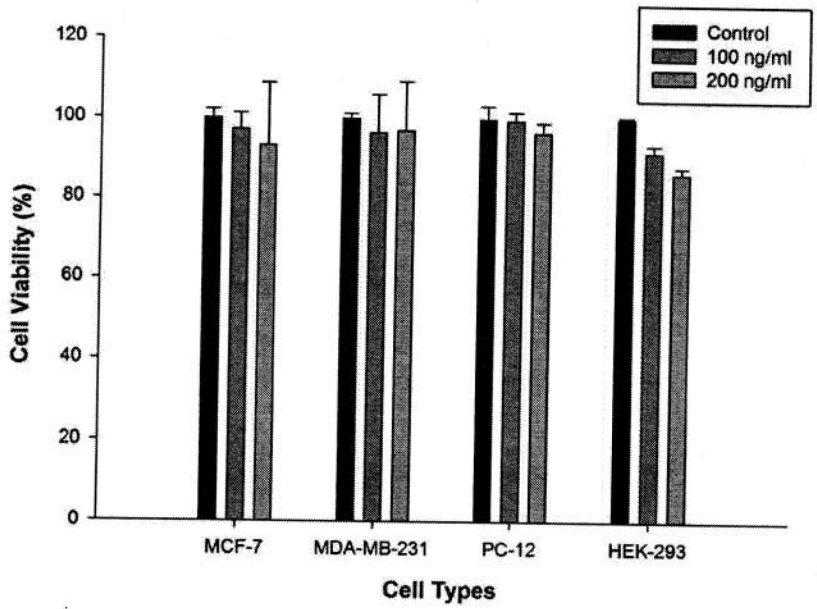


Fig 5B. Assessment of cytotoxicity of α- Cobrotoxin against various cell lines. The values are mean ± SD of triplicate determinations. The survival of treated cells did not differ from the control cells ($p>0.05$).

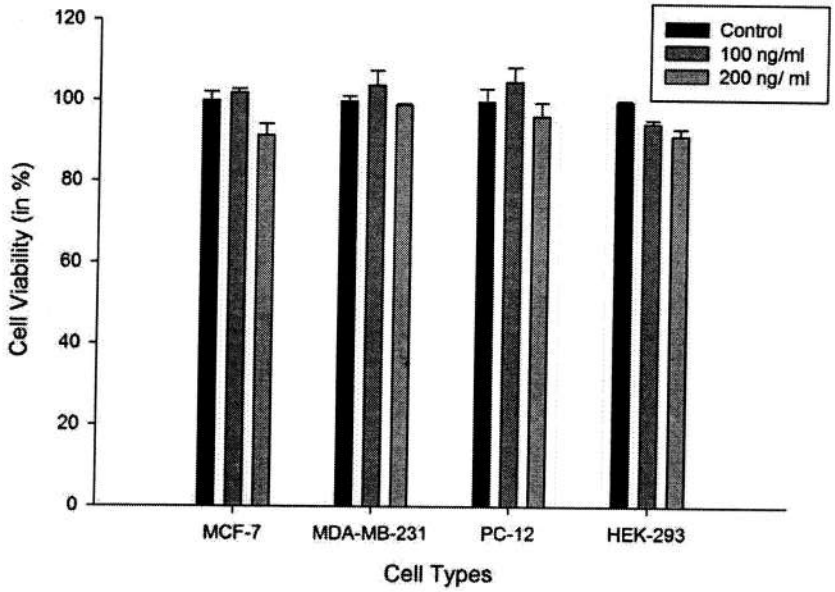


Fig 5C. Cytotoxicity screening of α-elapitoxin-Nk2a using MTT assay for various cell lines. The values are mean ± SD of triplicate determinations.

C. The receptor-ligand binding study: Investigation of interaction of above purified proteins (RVV NGF and cobra venom neurotoxins) with cell surface TrkA receptor highly expressed in surface of normal (PC-12) and malignant (breast cancer MCF-7) cells.

The binding of alpha-neurotoxin/ RVV-NGF to TrkA receptor was determined by ELISA. Briefly, the PC-12 or MCF-7 cells (0.5×10^5) were grown in 6 well cell culture plate in DMEM at 37 °C, 5% CO_2, and then anti-TrkA, TrkB, TrkC or $p75^{NTR}$ primary antibody (1:1000 in DMEM) was added to each well. After incubation for 1 h at 37 °C, 5% CO_2 the media was discarded and the cells were washed with 1xPBS for three times. The PC-12 cells treated with 1X PBS only served as control. Thereafter, alpha-cobratoxin (100 ng/ml) was added to each well and incubated for 2 h at 37 °C, 5% CO_2. The cells were washed with 1X PBS and the binding of alpha-cobratoxin/ RVV-NGF to PC-12 cells / MCF-7 cells was determined by ELISA using anti-alpha-cobratoxin/ RVV-NGF antibodies (1:1000) purified from polyvalent antivenom and rabbit anti-horse IgG conjugated with horseradish peroxidase (HRP) (1: 2000) as the secondary antibody. The binding of alpha-cobratoxin or RVV-NGF to 1 X PBS-treated cells was considered as base value (100% binding) and other values were compared to that.

Pre-incubation of PC-12 or MCF-7 cells with anti-TrkA antibody resulted in marked inhibition of binding of alpha-cobratoxin/ RVV-NGF to these cells; however, blocking of TrkB, TrkC & p75NTR receptor with respective antibodies did not influence their binding to PC-12 or MCF-7 cells (Fig 6A,B).These results suggest that alpha-cobratoxin and RVV-NGF bind specifically to the TrkA receptor of the breast cancer cells suggesting their application as diagnostic tools to detect those cancer highly expressing these receptors as compared to control cells.

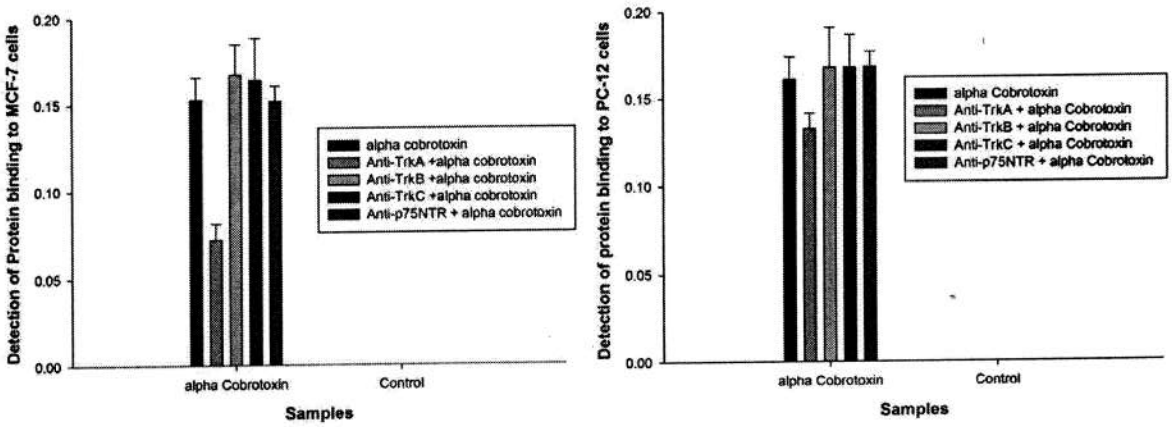


Fig 6A. Binding of alpha cobrotoxin to MCF-7 and PC-12 cells. The effect of anti-TrkA/B/C/ p75NTR antibody on ligand binding (alpha-cobrotoxin) to cellular receptor.

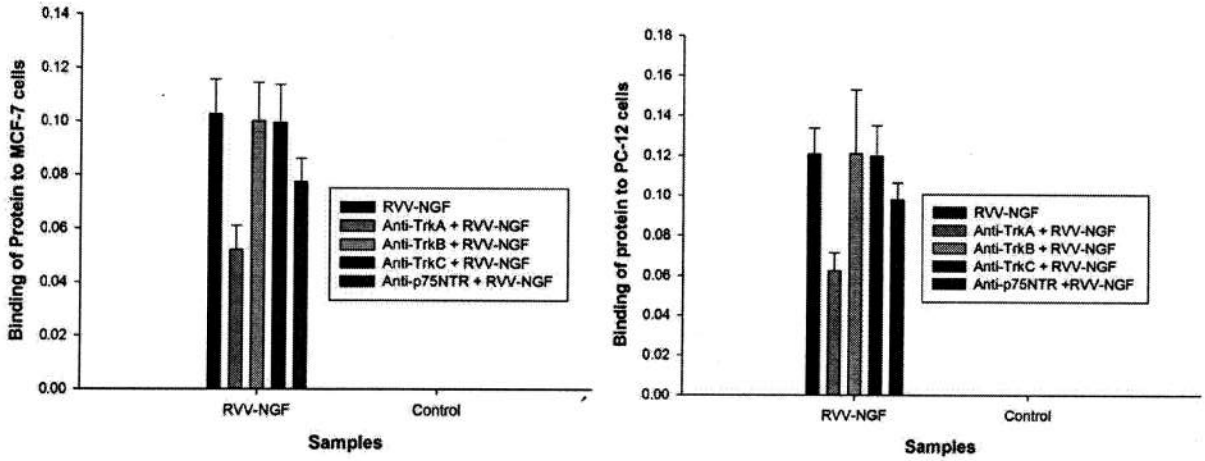


Fig 6B. Binding of RVV-NGF and 2S-NGF to MCF-7 and PC-12 cells. The effect of anti-TrkA/B/C/p75NTR antibody on ligand binding (NGF) to cellular receptor. The values are mean ± SD of triplicate determinations.

In another set of study it was observed that pre-incubation of PC-12 cells with anti-TrkA antibody or with K252a (chemical inhibitor of TrkA) resulted in abrogation of neuritogenesis potency of RVV-NGF and alpha-cobrotoxin (Fig 6C); however, pre-incubation of PC-12 cells with anti-TrkB/TrkC antibodies did not influence the RVV-NGF/ alpha-cobrotoxin-induced neuritogenesis in PC-12 cells suggesting specific binding of RVV NGF/ alpha-cobrotoxin to TrkA receptor subtype (Fig 6C).

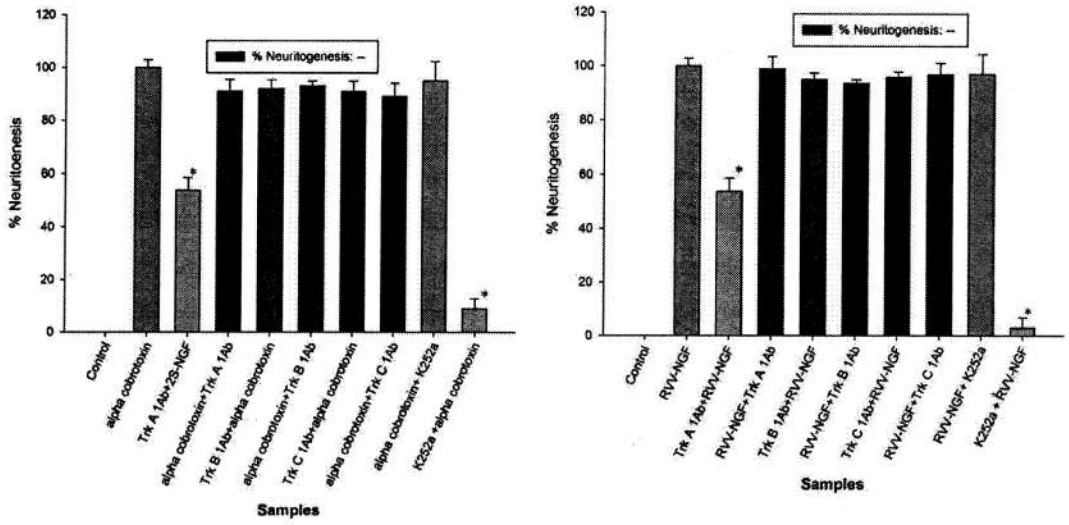


Fig 6C. Inhibitory effect of Anti-TrkA antibody and K252a on neuritogenesis induced by RVV NGF and alpha Cobrotoxin in PC-12 cells. Values are mean ± SD of three determinations. Significance of difference with respect to control, *p< 0.01.

10. Brief progress report (Russian Partner)

10 A. Synthesis of fluorescence CdSe quantum dots and labeling/conjugation of purified alpha-neurotoxins and NGF with functional nanoparticles as reporter groups.

For synthesis of CdSe/ZnS nanoparticles, the mixture of 0.0127 g (0.1 mole) of CdO and 0.1140 g (0.4 mole) of oleic acid was heated to 150° C with stirring under nitrogen. After complete dissolvution of CdO, the mixture was cooled to room temperature. Then, 1.94 g of octodecene was added, the mixture was heated to 320° C and maintained until the solution became clear. Selenium precursor is prepared by dissolving 0.079 g (1 mol) of Se in 0.238 g (1.18 mol) trioctylphosphine and 1.681 g (6.9 mol) octodecylamine (ODA). The obtained precursor is introduced into the reaction flask and solution obtained is kept for particle growth. Particle growth was stopped by introducing hexane into the reaction flask. The insoluble materials are separated by centrifugation and washed with methanol.

Building of ZnS shell was carried out at a constant temperature under a nitrogen atmosphere. To build up the shell zinc sulfide, zinc and sulfur precursors are prepared. The precursor of sulfur (0.1 mol) was prepared by dissolving 0.064 g of sulfur (0.002 mol) in 20 mL of ODE at 150 °C under a nitrogen atmosphere. Precursor of zinc (0.1 mol) was prepared by dissolving 0.275 g of zinc acetate (0.0015 mol) in a mixture of 4 ml and 11 ml ODE and OGE at 120 °C under argon. Zinc precursor was stored under nitrogen. For ZnS sell building mixture of sulfur precursor and OGE were placed in a flask purged with nitrogen for 20 min, then zinc precursor and CdSe in hexane were added. The temperature of the mixture was raised to 140 ° C, the mixture was kept at this temperature for 2 hours with constant stirring using a magnetic stirrer and then cooled. To the resulting solution were added 2 parts of acetone by volume, and the mixture was centrifuged at 4000 rev/min for 10 min. The resulting precipitate was washed with acetone, stabilized by hydrophobic surface ligands and dispersed in hexane at low concentration. The adsorption and fluorescence spectra of QDs obtained are shown on Fig. 7.

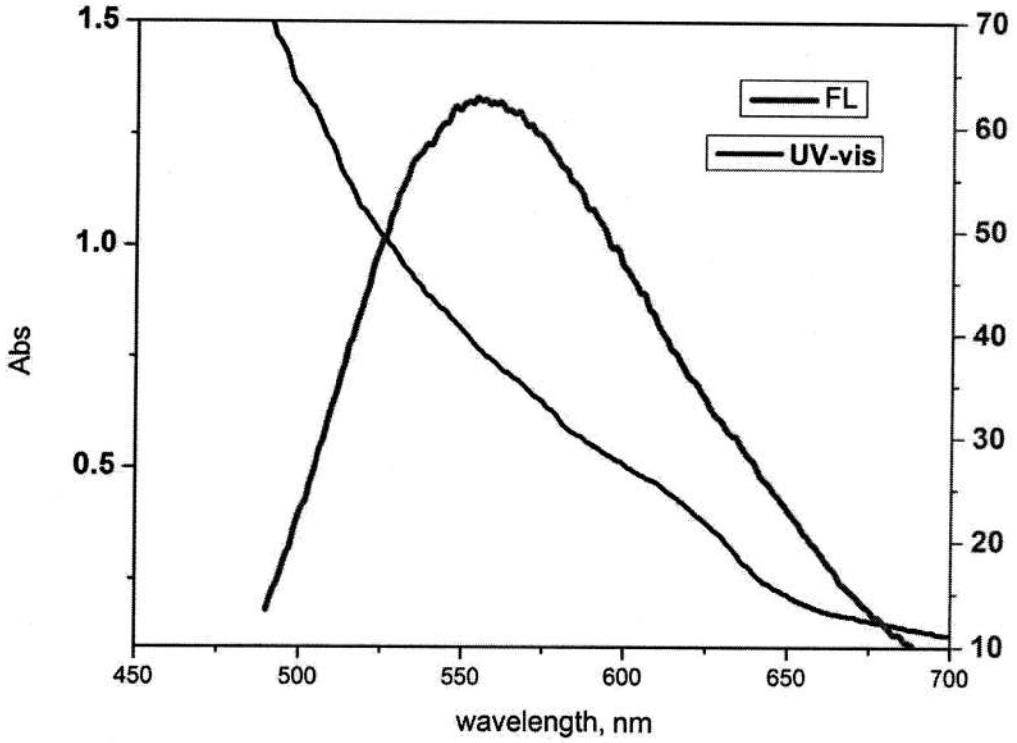


Figure 7. The adsorption (UV-vis) and fluorescence (FL) spectra of QDs

QDs functionalization using glutathione

To obtain water-soluble nanocrystals, a sample of CdS/ZnS (152 mg) was dissolved in 300 µl of hexane (506 mg/ml). Then 50 µl of the resulting solution was added to 150 µl of tetrahydrofuran (THF) and sonicated for 5 minutes. A portion of 20 mg of glutathione was dissolved in 200 ul of mixture of THF and water (1:1) (100 mg/ml). 130 µl of the resulting glutathione solution was added to a solution to CdS/ZnS in hexane/THF. The mixture was kept at 60 °C for 5 minutes and then centrifuge for 5 minutes at 13000 rev/min to completely precipitate modified CdS/ZnS nanocrystals. The organic and aqueous layers are removed, the precipitate was carefully washed with water, and dried at room temperature. To the dry residue 30 µl of water and 10 ul of an aqueous solution of tetrapropylammonium hydroxide were added. Thus, CdS/ZnS nanoparticles were obtained in aqueous phase at a slightly alkaline pH.

For the synthesis of conjugates glutathione modified CdS/ZnS quantum dots with toxins the latter were dissolved in water at a concentration of 10 mg/ml. Water soluble carbodiimide was used as activator of free carboxyl groups of glutathione. To the aqueous solution of glutathione-

modified quantum dots 25 µl of a solution of EDC and sulfo-NHS was added and the mixture was incubated at room temperature for 10 minutes. Then 50 µl of protein (alpha-cobrotoxin/ RVV-NGF) solution was added, the mixture was incubated for 60-70 min and separated by gel-filtration on Sephacel S-300. The separation profile is shown on Fig. 8.

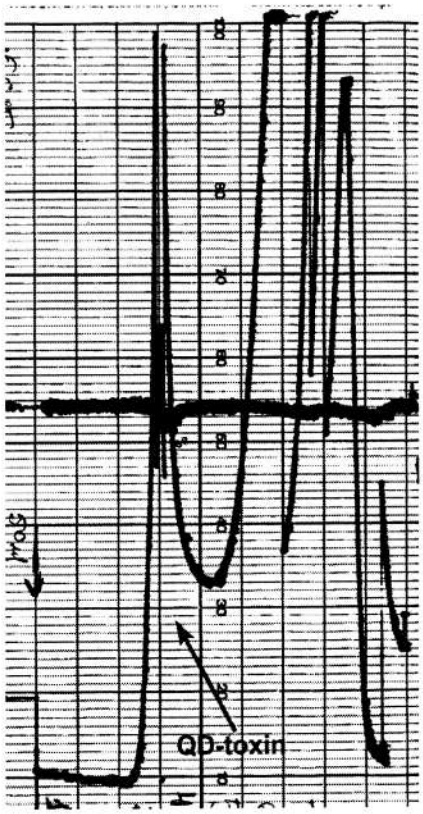


Figure 8. Separation of conjugates of QDs with toxin by gel-filtration on Sephacel S-300 column.

10B. Assessment of cytotoxicity of functionalized fluorescence nanoparticles conjugated with NGFs, short neurotoxin 1, and alpha-elapitoxin-Nk2a against breast cancer cells.

The study of cytotoxicity of fluorescent nanocrystals to be used for endogenous detection of cancer in various cell lines was carried out by MTT-assay. Cytotoxicity (ability of chemicals in damaging tissue/cell) was determined in *in-vitro* conditions against specific target cells viz. PC-12 and MCF-7, which represent a neuroendocrine tumor medullary cells of adrenal rat and adenocarcinoma cells of human breast cancer, respectively.

Rat pheochromocytoma PC-12 cells were cultured at 37 °C and 5% CO_2 in DMEM or RPMI 1640 medium (Sigma, USA) containing 10% fetal bovine serum (Hyclone) and 2 mM glutamine.

For studies, cells were seeded into 96-well plate (Corning, USA) at a density 10-50 $x10^3$ cells per well (60-70% of the monolayer). Human breast adenocarcinoma MCF-7 cells were cultured at 37 °C and 5% CO_2 in DMEM medium with sodium pyruvate (Sigma,, USA) containing 10% fetal bovine serum (Hyclone), 2 mM glutamine, 40 µg / ml Gentamicin and 10 µg / ml insulin. Cells at a density of 5×10^4 were seeded in 35 mm Petri dishes in a volume of 2 ml per dish.

Fig. 9 shows the absorption spectra in visible region of fluorescence nanoparticles used for cytotoxicity measurements.

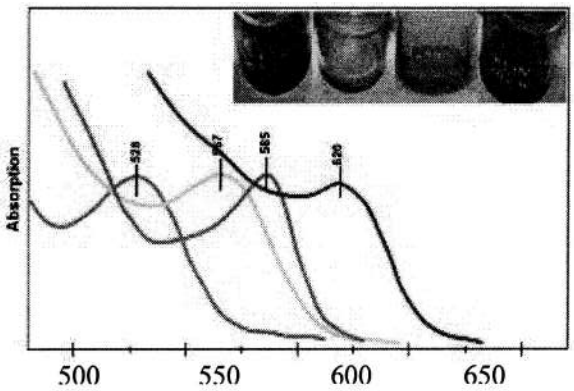


Fig. 9. Absorbance spectra in visible region of fluorescence nanoparticles used for cytotoxicity measurements.

Solutions containing respective concentrations of compounds were added into wells containing cells and incubation continued for another 48 h at 37 °C. The cells treated with medium only served as control. The cell cytotoxicity was assayed according to MTT based method. Data were expressed as a percentage of control (untreated) cells and the values were taken as mean ± SD of four replicates.

In total four different nanoparticle preparations have been used. Their effects on the survival of MCF-7 and PC12 cell lines studied. The results obtained are presented in Figs. 10 to 11. It was observed that MCF-7 cells were more resistant to the action of fluorescent nanoparticles than PC-12 cells.

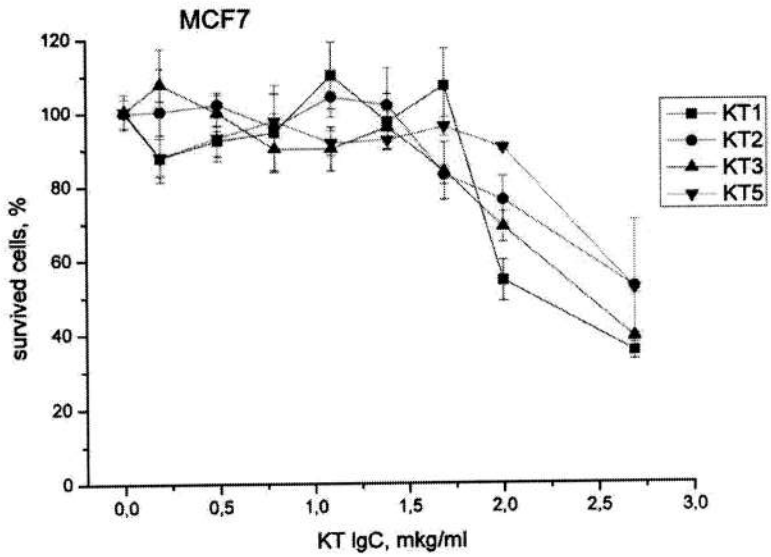


Figure 10A: Influence of fluorescent nanoparticles functionalized with glutathione on the survival of the MCF-7 cell line. Values are mean ± SD of four replicates.

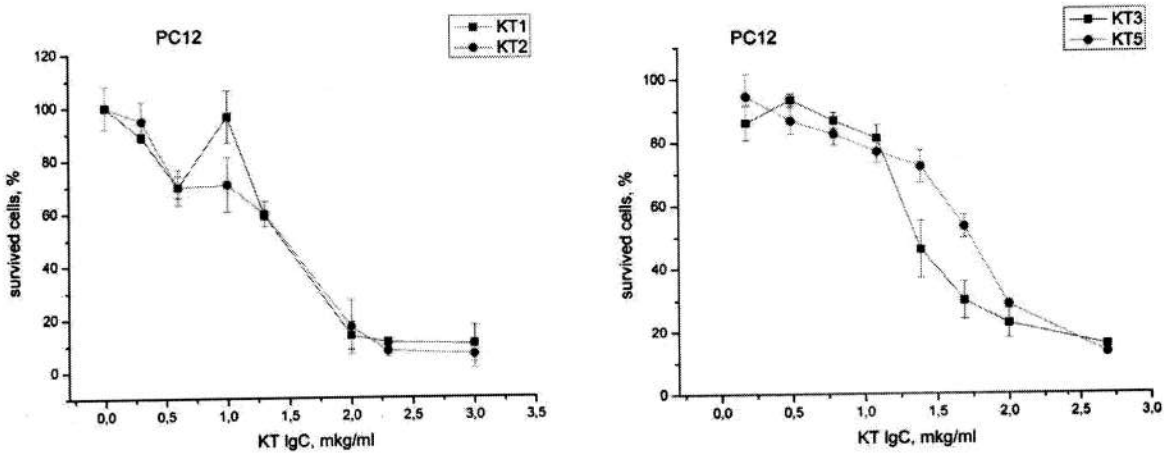


Figure 10B: Influence of fluorescent nanoparticles functionalized with glutathione on the survival of the PC-12 cell line. Values are mean ± SD of four replicates.

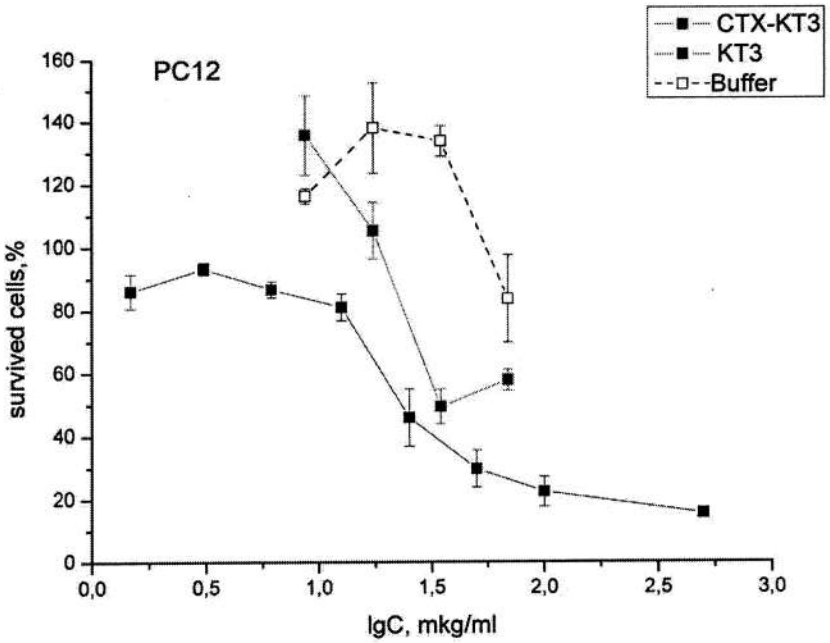


Figure 11A. Influence of alpha-cobratoxin conjugate with fluorescent nanoparticles on the survival of PC-12 cells. Values are mean ± SD of four replicates.

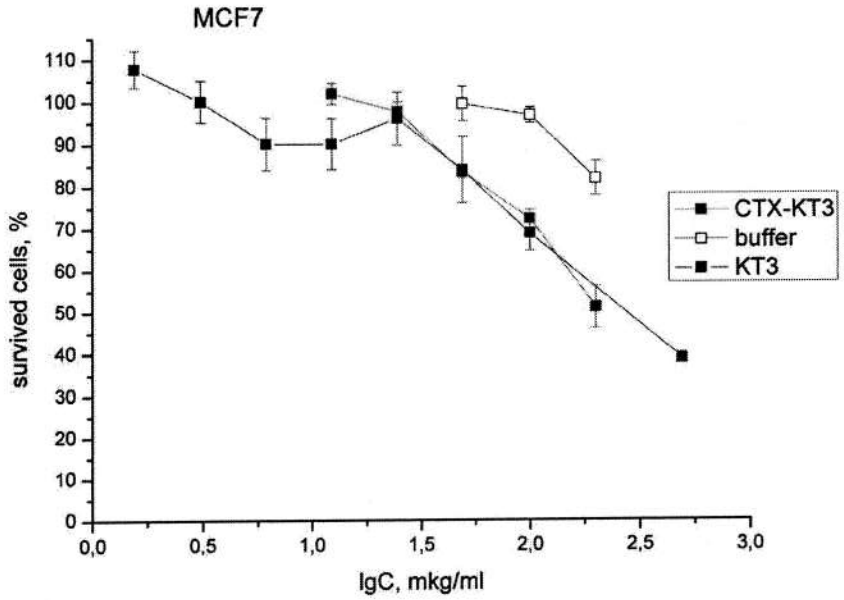


Figure 11B. Influence of alpha-cobratoxin conjugate with fluorescent nanoparticles on the survival of MCF-7 cells. Values are mean ± SD of four replicates.

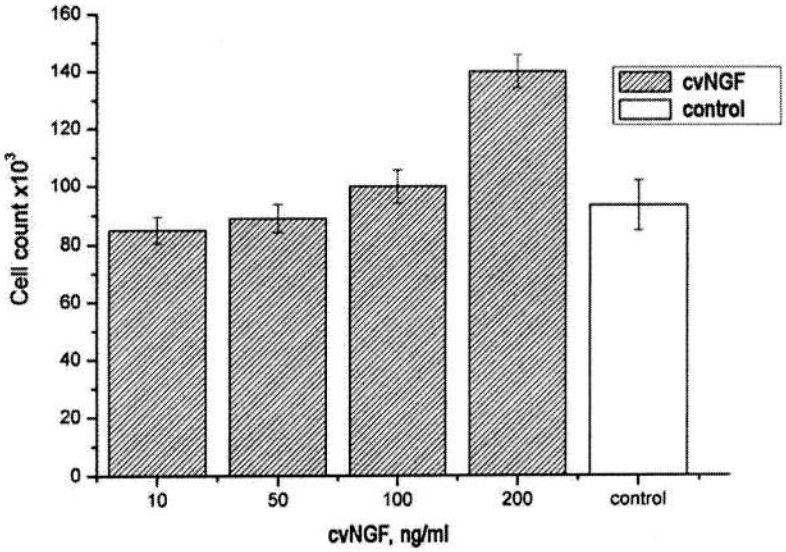
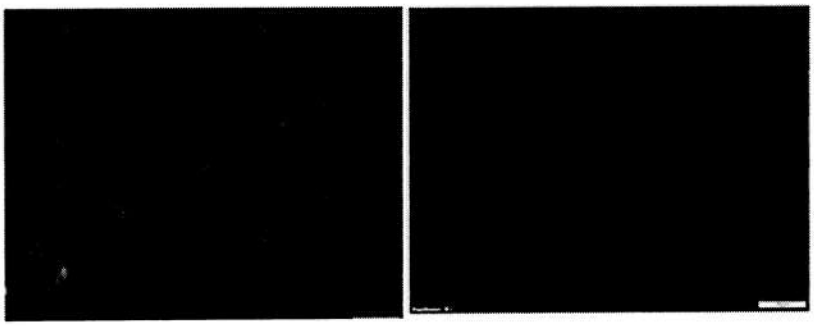


Fig. 11C: Effect of cobra venom nerve growth factor conjugated fluorescent nanoparticles on survival of MCF-7 cells. Values are mean ± SD of four replicates.

10C. Investigation of interaction of nanoparticles conjugated toxins with receptors (the receptor-ligand binding study) at the level of cells and tissue of normal as well as of malignant origin.

The binding of nanoparticles (CdSe) conjugated toxins (alpha-neurotoxins and NGF) to MCF and PC-12 cells was studied by fluroscence microscope. The PC-12 / MCF-7 cells (0.5 x 10^5) were grown in 96-well culture plate at 37 °C in a 5% CO_2 incubator for 24 h. The old media was replaced with fresh media containing 10% horse serum (in PBS-T containing 1% BSA) for 30 min at room temperature. The wells were washed with 1X PBS for three times and the cells were treated with 100 ng/ml of nanoparticles (CdSe) conjugated toxins / 1X PBS (untreated control), and grown for 60 min at 37 °C, 5% CO_2 followed by washing the cells with PBS-T (three times for 10 min each). The fluorescence intensity was determined by excitation at 568 nm and emission spectrum was recorded at 600 nm in a fluorescence microplate reader (Varioskan LUX, ThermoFisher scientific, USA).

The CdSe- conjugated toxins fluoresces bright green due to binding of toxins (alpha-cobrotoxin/ NGF) to trkA receptor of the treated cells (Figs. 12A, B).



A B.

Fig 12. Immunofluorescence detection of CdSe conjugated **A**. alpha-neurotoxin, **B**. RVV-NGF in PC-12 cells.

Receptor binding studies with alpha7 nicotinic acetylcholine receptor

Conjugate of alpha-neurotoxin with CdSe quantum dots were used for visualization of human alpha7 nicotinic acetylcholine receptors heterologously expressed in GH4CI pituitary adenoma cells. When the conjugate was irradiated with light wavelength of 530-550 nm, it resulted in fluorescence of cells due to binding of conjugate of alpha-neurotoxin with CdSe quantum dots with membrane receptors (Figs. 13A-C).

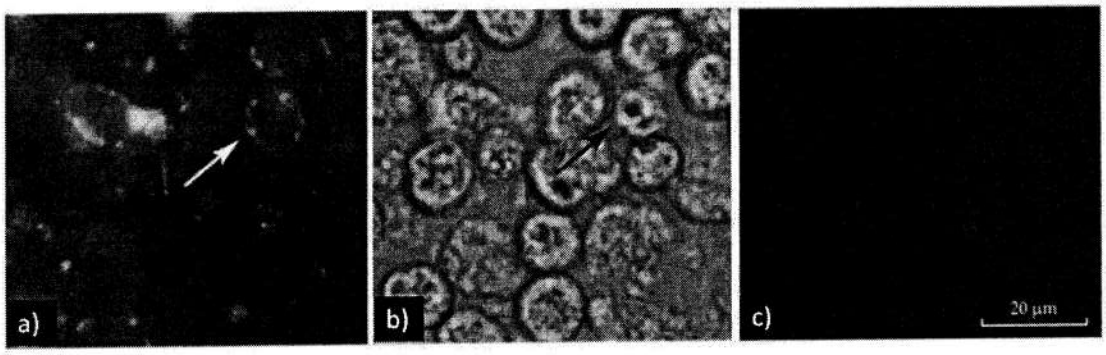


Fig 13 : Labeling of pituitary adenoma cells with the α-cobratoxin conjugated to QDs. (**A**) Fluorescence intensity of cells after incubation with the conjugate of α-cobratoxin with QDs (the white arrow shows the plasma membrane staining). (**B**) Image of cells obtained by bright-field microscopy in transmitted light (the black arrow shows the same cell as in panel (a) when photogrphed in transmitted light). (**C**) Fluorescence of the control cells without the addition of the conjugate.

List of referecnes:

Dutta, S., Chanda, A., Kalita, B.,Islam, T., Patra, A. and Mukherjee, A. K. (2017). "Proteomic analysis to unravel the complex venom proteome of eastern India Naja naja: Correlation of venom composition with its biochemical and pharmacological properties." J Proteomics 156: 29-39.

Kalita, B., Patra, A. and Mukherjee A. K., (2017). "Unraveling the Proteome Composition and Immuno-profiling of Western India Russell's Viper Venom for In-Depth Understanding of Its Pharmacological Properties, Clinical Manifestations, and Effective Antivenom Treatment." J Proteome Res 16(2): 583-598.

Mukherjee, A. K., Dutta, S., Kalita, B., Jha, D. K., Deb, P., and Mackessy, S. P., (2016). "Structural and functional characterization of complex formation between two Kunitz-type serine protease inhibitors from Russell's Viper venom." Biochimie 128-129: 138-147.

Mukherjee, A. K. and Mackessy, S. P., (2013). "Biochemical and pharmacological properties of a new thrombin-like serine protease (Russelobin) from the venom of Russell's Viper (Daboia russelii russelii) and assessment of its therapeutic potential." Biochim Biophys Acta 1830(6): 3476-3488.

Mukherjee, A. K., Saviola, A. J., Burns, P. D., and Mackessy, S. P., (2015). "Apoptosis induction in human breast cancer (MCF-7) cells by a novel venom L-amino acid oxidase (Rusvinoxidase) is independent of its enzymatic activity and is accompanied by caspase-7 activation and reactive oxygen species production." Apoptosis 20(10): 1358-1372.

Annexure I:

List of publications from this project:

1. Utkin, Y. N., Cherepakhin, I. Y., Kryukova, E. V., Shelukhina, I. V., Makarova, Y. V., Kasheverov, I. E., **Mukherjee. A. K.**, Kuznetsov, D. V. (2017). Conjugates of α-Cobratoxin with CdSe Quantum Dots: Preparation and Biological Activity. **In *Nano Hybrids and Composites*** 13, 3-8.
2. Makarova, Y. V., Shelukhina, I. V., **Mukherjee, A. K.**, Kuznetsov, D. V., Tsetlin, V. I., & Utkin, Y. N. (2017, July). Detection of human neuronalα7 nicotinic acetylcholine receptors by conjugates of snake α-neurotoxin with quantum dots. **In *Doklady Biochemistry and Biophysics*** 475, No. 1,253-255.

Manuscript under preparation:

1. Purification and characterization of nerve growth factor isoforms from Indian Russell's Viper (*Daboia russelii*) venom and elucidation of their binding to TrkA receptor of breast cancer cells.

Dated: 30 January, 2018

(A. K. Mukherjee)

Appendix-B[i]

Utilization Certificate

(For the financial year 1st April 2017-18th September 2017)

1	Title of the Project/Scheme	Application of snake venom toxins labeled with functionalized nanoparticles for detecting endogenous targets in cells and ex vivo tissues with prospects for the development of novel diagnostic and therapy tools
2	Name of the Organization	Department of Molecular Biology & Biotechnology, Tezpur University.
3	Principal Investigator	Prof. Ashis K. Mukherjee
4	Deptt. of Biotechnology sanction order No. & date of sanctioning the project	Sanction order no.- DBT/IC2/Indo-Russia/2014-16/03, Dated- 19-09-2014
5	Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given	Rs. 225168.00
6	Amount received from DBT during the financial year (2017-2018)	Rs. 0.00
7	Other receipts/interest earned, if any, on the DBT grants	Rs. 5046.00 (Interest receive for the financial year 2017-2018)
8	Total amount that was available for Expenditure during the financial year (6+7+8) :	Rs. 230214.00
9	Actual expenditure during the financial year (2017-2018):	Rs. 76262.00
10	Unspent balance refunded, if any *(Please give details of cheque No. etc.):*	Rs. 1,53,558.00 (DD No. 034426; dated 29-03-18) + Rs. 394.00 (DD No. 036541 ; dated 13/6/18)
11	Amount allowed to be carried forward to the next financial year vide letter No. & date.	NA

18/06/18

Joint Registrar
Tezpur University

1. Certified that the amount of ₹ 76262.00 (Rupees seventy six thousand two hundred and sixty two only) mentioned against col. 9 has been utilized on the project/scheme for the purpose for which it was sanctioned and that the balance of ₹ 1,53,952.00 (Rupees One lakh fifty three thousand nine hundred fifty two only) remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____).
2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Orders for chemicals were placed after T&PC approval of price.
2. Stock entry of chemical, consumable & contingency items etc.
3.
4.
5.

(Signature of Principal Investigator)

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular Biology & Biotechnology
Tezpur University
Tezpur-784028, Assam

(Signature of Finance Officer)

Joint Registrar
Tezpur University

(Signature of Head of the Institute)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

1. Certified that the amount of ₹ 76262.00 (Rupees seventy six thousand two hundred and sixty two only) mentioned against col. 9 has been utilized on the project/scheme for the purpose for which it was sanctioned and that the balance of ₹ 1,53,952.00 (Rupees One lakh fifty three thousand nine hundred fifty two only) remaining unutilized at the end of the year has been surrendered to Govt. (vide No. _____ dated _____).
2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Orders for chemicals were placed after T&PC approval of price.
2. Stock entry of chemical, consumable & contingency items etc.
3.
4.
5.

11/6/2018

(Signature of Principal Investigator)

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular
Biology & Biotech
Tezpur University
Tezpur- 784028 Assam

18/6/18

(Signature of Finance Officer)
Joint Registrar
Tezpur University

21/6/18

(Signature of Head of the Institute)
Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

1. Certified that the amount of ₹ 27,64,756.00 (Rupees Twenty seven lakhs sixty four thousand seven hundred and fifty six only) mentioned against col. 9 has been utilized on the project/scheme for the purpose for which it was sanctioned and that the balance of ₹ 1,53,558.00 (Rupees one lakh fifty three thousand five hundred fifty eight only) and ₹ 394.00 (Rupees Three hundred and ninety four only) remaining unutilized at the end of the project has been surrendered to Govt. (DD No. 034426; dated 29-03-18) and (DD No. 036541 ; dated 13/6/18).
2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilized for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Orders for chemicals were placed after T&PC approval of price.
2. Stock entry of chemical, consumable & contingency items etc.
3. Appointment of JRF following the DBT guidelines after floating the advertisement.
4.
5.

11/6/2018

(Signature of Principal Investigator)

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular
Biology & Biotechnology
Tezpur University (Central)
Tezpur- 784028, Assam

18/06/18

(Signature of Finance officer)

Joint Registrar
Tezpur University

11/6/18

(Signature of Head of the Institute)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

Statement of Expenditure referred to in para 9 of the Utilisation Certificate

Appendix-A

Showing grants received the Department of Biotechnology and the expenditure incurred during the period of 1st April 2017-18th September, 2017. **(Amount in Rupees)**

Sl. No.	Heads	Grant receive from DBT for the year (2017-2018)	Unspent balance during the year (2016-2017)	Total (2+3)	Expenditure incurred during the year (1.4.16-31.3.17)	Balance
	1	2	3	4	5	6
	I. Recurring					
(i)	Manpower	0	67200	67200	60000	7200
(ii)	Consumables and analysis charge	0	6074	6074	0	6074
(iii)	Travel-Domestic	0	2770	2770	1420	1350
	Travel-International	0	104260	104260	0	104260
(iv)	Contingency	0	14847	14847	14842	5
(v)	Overheads	0	0	0	0	0
(vi)	Maintenance	0	21750	21750	0	21750
	Interest earned	5046	8267	13313		13313
Total		**5046**	**225168**	**230214**	**76262**	**153952**

1/11/2018

(Signature of Principal Investigator)

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular Biology & Biotechnology
Tezpur University (Central)
Tezpur- 784028, Assam

2/11/18

(Signature of Head of the Institute)
Registrar
Tezpur University

(Signature of Finance officer)
Joint Registrar
Tezpur University

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. **Title of the Project** : Application of snake venom toxins labeled with functionalized nanoparticles for detecting endogenous targets in cells and ex vivo tissues with prospects for the development of novel diagnostic and therapy tools.
2. **Sanctioned Project Cost:** Rs. 45.60 Lakhs (Rupees Forty five lakhs and sixty thousand only)
3. **Revised cost, if any** : Nil
4. **Duration of the project:** Three Years (2014-2017)
5. **Sanction Order No. & Date:** Sanction order no. DBT/IC2/Indo-Russia/2014-16/03, Dated- 19-09-2014
6. **Date of commencement of Project:** 19th September 2014.
7. **Extension, if any:** Nil
8. **Date of completion of project:** 18th September, 2017

❖ **Details of grant, expenditure and balance**

Sl. No.	Heads	Total sanctioned (Rs.)	Year wise releases made (in Rs.)					Year wise expenditure incurred (in Rs.)					
			1st yr (19.9.14-31.3.15)	2nd yr (01.04.15 - 31.03.16)	3rd yr (01.04.16-31.03.17)	4th yr (01.04.17-18.09.17)	Total (Rs.)	1st yr (19.9.14-31.3.15)	2nd yr (01.04.15-31.03.16)	3rd yr (01.04.16-31.03.17)	4th yr (01.04.17-18.09.17)	Total (Rs.)	Balance (Rs.)
(i)	Manpower	660000.00	211200	0	1,36,493.00	0	3,47,693.00	30,428.00	1,06,065.00	1,44,000.00	60,000.00	3,40,493.00	7,200.00
(ii)	Consumable and Analysis charge	1800000.00	6,00,000.00	0	6,00,000.00	0	12,00,000.00	1,22,974.00	4,76,272.00	5,94,680.00	0	11,93,926.00	6,074.00
(iii)	Travel - Domestic	150000.00	50,000.00	0	50,000.00	0	1,00,000.00	15,190.00	34,503.00	47,537.00	1,420.00	98,650.00	1350.00
	Travel - International	600000.00	0		3,00,000.00	0	3,00,000.00	0	0	1,95,740.00	0	1,95,740.00	1,04,260.00
(iv)	Contingency	300000.00	1,00,000.00	0	1,00,000.00	0	2,00,000.00	22,487.00	84,720.00	77,946.00	14,842.00	1,99,995.00	5
(v)	Maintenance	900000.00	5,00,000.00		1,41,370.00	0	6,41,370.00	0	4,57,702.00	1,78,250.00	0	6,35,952.00	5,418.00
(vi)	Overheads	150000.00	50,000.00	0	50,000.00	0	1,00,000.00	31,250.00	17,797.00	50,953.00	0	1,00,000.00	0
	Interest earned		13,965.00	2,367.00	8,267.00	5,046.00	29,645.00	0	0	0	0	0	29,645.00
	Totals	4560000	15,25,165.00	2,367.00	13,86,130.00	5,046.00	29,18,708.00	2,22,329.00	11,77,059.00	12,89,106.00	76,262.00	27,64,756.00	1,53,952.00

16/2018

(PROJECT INVETIGATOR)

Prof. A.K. Mukherjee, Ph.D., D.Sc.
Department of Molecular
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Tezpur University (Central)
Tezpur- 784028, Assam

21/6/18

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

21/6/18

(FINANCE OFFICER)
Joint Registrar
Tezpur University