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Antiproliferative effect of T/Tn specific *Artocarpus lakoocha* agglutinin (ALA) on human leukemic cells (Jurkat, U937, K562) and their imaging by QD-ALA nanoconjugate

Urmimala Chatterjee · Partha Pratim Bose · Sharmistha Dey · Tej P. Singh · Bishnu P. Chatterjee

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Abstract T/Tn specificity of *Artocarpus lakoocha* agglutinin (ALA), isolated from the seeds of *A. lakoocha* (Moraceae) fruit and a heterodimer (16 kD and 12 kD) of molecular mass 28 kD, was further confirmed by SPR analysis using T/Tn glycan containing mammalian glycoproteins. N-terminal amino acid sequence analysis of ALA showed homology at 15, 19–21, 24–27, and 29 residues with other lectin members of Moraceae family *viz.*, *Artocarpus integrifolia* (jacalin) lectin, *Artocarpus hirsuta* lectin, and *Maclura pomifera* agglutinin. It is mitogenic to human PBMC and the maximum proliferation was observed at 1 ng/ml. It showed an antiproliferative effect on leukemic cells, with the highest effect toward Jurkat cells (IC_{50} 13.15 ng/ml). Synthesized CdS quantum dot-ALA nanoconjugate was employed to detect the expression of T/Tn glycans on Jurkat, U937, and K562 leukemic cells surfaces as well as normal lymphocytes by fluorescence microscopy. No green fluorescence was observed with normal lymphocytes indicating that T/Tn determinants, which are recognized as human tumor associated structures were cryptic on normal lymphocyte surfaces, whereas intense green fluorescent dots appeared during imaging of leukemic cells, where such determinants were present in unmasked form. The above results indicated that QD-ALA nanoconjugate is an efficient fluorescent marker for identification of leukemic cell lines that gives rise to high quality images.

Keywords ALA · *Artocarpus lakoocha* agglutinin · Surface plasmon resonance · Leukemic cells · Quantum dot · Nanoconjugate

Abbreviations

ALA	*Artocarpus lakoocha* agglutinin
BSM	Bovine submandibular gland mucin
CdS	Cadmium sulphide
ELLSA	Enzyme - linked lectinsorbent assay
ESI-MS	Electron spray ionization mass spectrometry
FPLC	Fast protein liquid chromatography
HBS	Hepes buffered saline
HEPES	*N*-(2-hydoxyethyl) piperizine-*N*′-(2-hydroxypropane sulfonic acid)
HSM	Hamster submaxillary mucin
PBMC	Peripheral blood mononuclear cells
PVDF	Polyvinylidene difluoride

U. Chatterjee · P. P. Bose · B. P. Chatterjee (✉)
Department of Biological Chemistry,
Indian Association for the Cultivation of Science,
Jadavpur, Kolkata 700 032, India
e-mail: cbishnup@gmail.com

S. Dey · T. P. Singh
Department of Biophysics,
All India Institute of Medical Sciences,
Ansari Nagar, New Delhi 110029, India

Present address:
B. P. Chatterjee
Department of Biotechnology,
West Bengal University of Technology,
BF 142, Sector 1, Salt Lake,
Kolkata 700 064, India

Present address:
U. Chatterjee
Department of Anatomy, Physiology and Biochemistry,
Swedish University of Agricultural Science,
Uppsala, Sweden

Present address:
P. P. Bose
Department of Biochemistry and Organic Chemistry,
Uppsala University,
Uppsala, Sweden

Q-ToF	Quadrupole-time of flight
QD	Quantum dot
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
2 ME	2-Mercaptoethanol

Introduction

Lectins comprise a structurally diverse class of proteins or glycoproteins other than antibodies or enzymes that bind specifically and reversibly to mono- and oligosaccharides [1]. They are ubiquitous in the biosphere and have been isolated from viruses, bacteria, fungi, plants and animals. Because of their well defined specificity they serve as valuable reagents in studies of biochemistry, cell biology, hematology, immunology, glycobiology, and oncology and have enormous applications in biomedical researches including cancer research [2]. Lectins aid to differentiate between the malignant and normal cells based on their agglutination pattern. This is due to altered glycosylation on cell surface associated with malignancy, its progression and metastasis [3, 4]. They can be employed for the detection of glycan changes in certain disease processes which involve fucosylation, increased sialylation and increased branching of complex carbohydrates. Recognition of these altered structural profiles of glycans by lectins provides valuable disease biomarkers [5–8]. Recent development in quantum dot (QD) nanotechnology have resulted in the introduction of new fluorescent immunocytochemical probes, in which QDs are covalently coupled to lectins. These were used for ultrasensitive biological imaging and analysis using fluorescent microscopy and/or flow cytometry [9–11].

Previously, we isolated a lectin from the seeds of *Artocarpus lakoocha* fruit, a plant belonging to Moraceae family, and its carbohydrate specificity was defined against monosaccharides and several T related disaccharides [12–14]. Further examination of its glycan affinity at macromolecular level by enzyme-linked lectinsorbant assay and binding-inhibition assay using mammalian glycotopes in macromolecules revealed its binding specificity to tumor-associated carbohydrate antigens GalNAcα1→Ser/Thr (Tn) and Galβ1-3GalNAcα1→Ser/Thr (Tα). It hardly cross-reacted with common glycotopes on glycoproteins, including ABH blood group antigens, Galβ1-3/4GlcNAc determinants, T/Tn covered by sialic acids and N-linked serum glycoproteins [15]. However, no biological property of this lectin was reported till now. The present study reports purification of this lectin by affinity repulsion chromatographic technique, some of its biological properties and the binding specificity toward T/Tn containing glycoproteins using surface plasmon resonance analysis. The present work also demonstrates a unique and simple technique based on cadmium sulphide nanoparticle (QD) tagged ALA, which was used as a sensitive probe for distinguishing leukemic cell lines, *viz*., Jurkat, U937 and K562 from normal lymphocytes.

Materials and methods

RPMI-1640 culture medium and fetal bovine serum (FBS) were purchased from GIBCO, USA. Hepes, sodium bicarbonate, Ficoll-Histopaque, 2-mercaptoethanol, penicillin, streptomycin, fungizone, glutamine, biotinamidocaproate-*N*-hydroxy-succinimide ester, antibiotin-HRP and trypan blue were purchased from Sigma, USA. [^{3}H] Thymidine was the product of New England Nuclear, USA. Tissue culture plates (96 well), petri dishes, culture bottles were procured from Axygen, Sweden. Asialo BSM, Tn containing glycopeptides (MW<3,000 Da) and T disaccharide (Galβ1→3GalNAc) were the kind gift of Prof. A. M. Wu, Chang-Gung University, Kwei-San, Taipe, Taiwan. Methyl-α-galactose (Me-α-Gal), Methyl-α-*N*-acetylgalactosamine (Me-α-GalNAc) were kindly obtained from Prof. N. Roy, Department of Biological Chemistry, Indian Association for the Cultivation of Science, Kolkata, India. Asialo glycophorin which has fifteen O-linked glycan of Tα (Galβ1→3GalNAc α1→Ser/Thr) lectin determinants and one N-linked carbohydrate residue was generously supplied by Prof. E. Lisowska (Institute of Immunology and Experimental Therapy, Wroclaw, Poland, and prepared according to Wu and Pigman [16]. Tn-glycophorin was prepared by removing galactose residue from asialo glycophorin by periodate oxidation and mild acid hydrolysis [17]. Fetuin and BSM (Sigma) were desialylated by 0.01M HCl at 80^0 C for 90 min. Small fragments and HCl were removed by extensive dialysis against H_2O.

Purification of *Artocarpus lakoocha* agglutinin

ALA was purified by a combination of two methods [12, 13] as reported earlier [15]. In brief, the saline extract of the seeds (20% *w*/*v*) was precipitated by 40% saturated ammonium sulfate with vigorous stirring for 3 h at 4°C. The resulting precipitate was dissolved in a minimum volume of saline and was subjected to 0.2% (*w*/*v*) rivanol (6, 9-diamino-2-ethoxy acridine lactate) precipitation. After removal of excess rivanol by precipitation with KBr, the supernatant was extensively dialyzed against saline overnight at 4°C. The dialyzed solution after concentration by YM 10 membrane was subjected to affinity chromatography on melibiose-agarose column (20 cm×1 cm). The unbound proteins were eluted with 10 mM PBS, pH 7.2 till A_{280} of effluents was less than 0.002. The bound protein was desorbed

with deionized water following the principle of affinity repulsion chromatography [18]. The active fractions were mixed and after concentration by YM 10 membrane was stored −20°C with 0.02% NaN_3 for further work. The minimum concentration of ALA for erythroagglutination was 3.57 ng/ml. Protein content of all the fractions were determined by Bradford method [19] using BSA as the standard.

Hemagglutination

The hemagglutinating activity of the purified agglutinin was determined by incubating a twofold serially diluted agglutinin solution (25 μl) in saline with an equal volume of 2% (*v*/*v*) heparinized normal human B erythrocytes suspension in saline for 30 min at room temperature. The normal erythrocytes were prepared according to Bhowal *et al*. [20]. Hemagglutination titer was defined as the reciprocal of the highest dilution showing visible hemagglutination.

PAGE, SDS-PAGE and molecular mass

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing condition was performed on 0.75 mm slab gel (10%) in acidic buffer system (β alanine/acetic acid, pH 4.3) according to Reisfled *et al*. [21]. The protein bands were visualized by Coomassie brilliant blue G-250 staining followed by destaining in 5% acetic acid.

SDS-PAGE was done on 12% polyacrylamide gel in Tris/glycine buffer pH 8.3 according to the method of Laemmli [22]. The sample was heated with 1% SDS in the presence or absence of 2-ME for 5 min at 100^0 C. The gel was stained with Coomassie brilliant blue, G-250. Molecular mass of the lectin was calculated according to the relative mobility with the Precision Plus Protein™ Standards.

The molecular mass of ALA was determined by gel filtration using Superose 6, 10/300 GL column in FPLC system (Amersham Biosciences, Sweden) at 4°C in TBS at a flow rate 24 ml/h. The molecular mass was calculated from the relative elution volume of different standard proteins of gel filtration kit (Sigma, USA). The molecular mass of ALA was also determined by ESI-MS using 5 μl/min flow rate of 100 pmol lectin solution (50:50:1; acetonitrile: water: formic acid) on Micromass Q-ToF Micro (Waters) mass spectrometer.

N-terminal amino acid sequencing

ALA was run on 12% SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membrane at 100 mA constant current for 18 h using 10 mM 3-cyclohexylamino-1-propane-sulfonic acid (CAPS), pH 11.0, containing 10% MeOH as the blotting buffer [23]. After transfer of the protein band to PVDF, the membrane was rinsed several times with Milli Q water and then saturated with 100% MeOH for 2 s. The blot was next stained with 0.1% Amido black in 40% MeOH containing 1% AcOH for 1 min and destained with 50% MeOH. The membrane was washed with several changes of water, air dried and the protein band was excised.

The N-terminal amino acid sequence of the electro-blotted ALA was done in a gas-phase protein sequencer (Shimadzu model PPSQ-21A) consisting of Edman reaction unit followed by HPLC and UV detection [24].

Glycan detection of ALA

ALA was electrophoresed on 10% SDS-PAGE gel and blotted onto nitrocellulose membrane, using transfer buffer 0.192 M glycine, 25 mM Tris pH 8.9, 20% (*v*/*v*) methanol at 100 mA constant current for 4 h [23]. The complete transfer of ALA bands was checked by staining with Ponceau S (0.2% Ponceau S in 1% acetic acid). The type of glycoform in ALA subunits was performed using DIG Glycan Differentiation Kit (Roche Applied Science) after transferring the lectin bands to NCM.

Binding studies of ALA with glycoproteins and glycans by surface plasmon resonance (SPR) analysis

The binding studies were carried out using the BIAcore 2000 SPR apparatus, (BIAcore AB, Uppsala, Sweden) at 25°C. The BIAcore apparatus is a biosensor-based system for real time specific interaction analysis [25]. The sensor chip CM5, surfactant P20, amine coupling kit containing *N*-hydroxysuccinimide (NHS), *N*-ethyl-*N*″-3(diethylamino-propyl) carbodimide (EDC) and ethanolamine hydrochloride used were supplied by BIAcore AB. After equilibration with 5 mM HEPES-buffered saline (pH 7.4) and 0.005% surfactant P20, the surface of the sensor chip was activated with a 1:1 mixture (70 μl) of 0.1 M NHS and 0.1 M EDC. ALA (200 μg/ml) was immobilized in 10 mM sodium acetate buffer (pH 4.8) at a flow rate of 5 μl/min for 30 min and unreacted groups were blocked by injection of 1.0 M ethanolamine (pH 8.5). The association rate constants were determined by passing the glycan solutions (5–20 nM) over the chip at a flow rate of 5 μl/min for 8 min. The dissociation rate constants were determined by passing HBS at a flow rate of 5 μl/min for 8 min and dissociation was followed by passing HBS at a flow rate of 5 μl/min for 10 min. After every cycle the sensor chip was regenerated by passing 100 mM HCl for 1 min. Kinetic parameters were calculated by BIA evaluation software version 3.0.

Isolation of human peripheral blood mononuclear cells

Blood from normal healthy individual taken in heparin was layered on Ficoll-Histopaque solution and centrifuged at

1,800 rpm for 30 min at room temperature. The PBMC were collected from the interface, washed thrice with RPMI-1640, and contaminating erythrocytes were depleted by hypotonic shock. The cells were suspended in RPMI-1640 supplemented with fetal bovine serum (10%), Hepes (25 mM), glutamine (200 μM), penicillin (100 IU/ml), streptomycin (100 μg/ml) and 0.5% fungizone [26]. The cellular viability was assessed by the trypan blue exclusion test. The percentage of cells that excluded the dye was enumerated in a hemocytometer under phase contrast microscope (Leitz, Diaplan).

Assay of mitogenic activity of ALA

The PBMC suspension (10^6 cells/ml, 0.1 ml) was cultured for 72 h at 37°C in humidified atmosphere of 5% CO_2 (Heraus) with different concentrations of ALA (0.01, 0.1, 1, 10, 100 and 1,000 ng/ml) in 96 well tissue culture plates in triplicate [27]. The wells that received only PBMC suspension were used as negative control and those with PBMC suspension along with ConA (3 μg/ml) were treated as positive control. To each well of the plates incubated for 72 h radioactive precursor, 1 μCi $[H]^3$ thymidine was added and the plates were incubated for 18 h. The cells were harvested onto GFC membrane by a cell harvester. The incorporated radioactivity was measured by liquid scintillation counter [28].

Antiproliferative activity of ALA on leukemic cell lines

The human leukemic cell lines Jurkat, U937 and K562 were obtained from National Centre for Cell Science, Pune. All cell lines were cultured and maintained in RPMI 1640 as described before. The cells attained 70% confluency within 48 h. The antiproliferative activity of ALA *in vitro* was determined as follows [29]. Cells (1×10^4/ ml) in their exponential growth phase were seeded into each well of 96 well culture plates (NUNC) and incubated for 3 h. Thereafter ALA (0.4, 4 and 40 ng/ml) was added and incubation was carried out for another 48 h. Radioactive precursor, 1 μCi $[H]^3$ thymidine was added to each well and incubated for 18 h. The cells were harvested onto GFC membrane by a cell harvester. The incorporated radioactivity was measured by liquid scintillation counter.

Synthesis of ALA conjugated cadmium sulphide nanoparticle

CdS-nanoparticle tagged ALA was prepared by arrested precipitation of CdS nanocrystals at 18°C in solution phase using ALA as the colloidal stabilizer. CdS nanocrystals were prepared as follows. 0.9 mg of $CdCl_2$ was dissolved in 100 ml (0.05 mM) of deionized water with vigorous stirring. 1 ml of 0.1 mM ALA solution (in deionized water) was added to the 1 ml stirred solution of $CdCl_2$ prepared above and the mixture was allowed to stir gently for another 15 min. Thereafter 20 μl of Na_2S, $9H_2O$ (0.1 M) solution in tris-buffer (20 mM, pH 8.2) was added to the previous solution in a stirring condition. The solution turned faint green due to *in situ* formation of lectin stabilized CdS quantum dot (QD). The formation of lectin bound CdS nanoparticle was ensured by the photoluminescence spectra, Transmission electron microscopy (TEM), Energy Dispersive X-ray analysis (EDX) and fluorescence microscopy. The activity of QD-ALA was determined by hemagglutination with human erythrocytes along with ALA.

Transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) was carried out using a JEOL JEM-2010 electron microscope to observe finer morphological details of the ALA - tagged quantum dots. The experiment was performed using a small amount of the aforementioned solution in tris-buffer on carbon-coated copper grid (300 mesh) by slow evaporation and subsequent vacuum drying at 18°C for 2 days. Images were taken at an accelerating voltage of 200 kV.

Fluorescence microscopy of the interaction of QD-ALA conjugate with cells

To examine the attachment of QD-ALA to various cells such as Jurkat, U937, K562 and normal PBMC, the cells were washed twice with 10 mM phosphate buffer saline (PBS), pH 7.4 and fixed with 4% paraformaldehyde in PBS for 10 min. The fixed cells were then blocked by 1% (*w*/*v*) BSA in PBS for 20 min and were separately incubated with 10 μl of QD-ALA solution for 1 h. Following incubation the cells were washed thoroughly twice with PBS. A drop of QD-ALA attached cell suspension was mounted on a glass slide covered with a cover slip and imaged immediately under fluorescence microscope.

Results

Purification of *A. lakoocha* agglutinin

Table 1 shows the purification scheme of *A. lakoocha* agglutinin. The crude seed extract on precipitation with 40% ammonium sulphate gave a protein of 18.2-fold purification with 80% yield. This upon treatment with rivanol achieved purification of 80-fold with 64% yield. Since the hemagglutination activity of ammonium sulphate precipitated fraction and rivanol precipitated lectin was inhibited by melibiose, affinity chromatography on meli-

Table 1 Purification scheme of *A. lakoocha* lectin[a]

Lectin Fraction	Total volume (ml)	Protein concen-tration (mg/ml)	Total protein (mg)	Total activity (HU)[b]	Specific activity (HU/mg)	Purification fold	Yield (%)
Crude extract	500	5.0	2500	512×10^3	205	1.0	100
Ammonium sulphate precipitated fraction	50	2.2	110	410×10^3	3,730	18.2	80.0
Rivanol precipitated lectin	20	1.0	20	328×10^3	16,400	80.0	64.0
Affinity purified lectin	2	0.152	0.304	262×10^3	862,000	4,205	51.2

[a] Data shown are mean of three experiments
[b] Hemagglutination unit (HU) is defined as minimum amount of protein (μg/ml) showing hemagglutination with normal human B erythrocytes

biose-agarose column was used for isolation of pure lectin (Fig. 1). The rivanol precipitated lectin was totally absorbed to the column, which was eluted with deionized water following the technique of affinity repulsion chromatography. The specific activity of the purified *A. lakoocha* agglutinin designated as ALA was 8,62,000 with 4,205-fold purification. The recovered activity of the purified protein was 51.2% and minimum concentration of the protein required for erythro-agglutination was 3.6 ng/ml.

The homogeneity of ALA was proved by polyacrylamide gel electrophoresis as it produced a single band in non-denaturing acidic gel (Fig. 2a). However, ALA by SDS-PAGE (12%) under denaturing condition with or without 2-ME, produced two bands at ~16 and ~12 kD respectively indicating that ALA was a heterodimeric lectin (Fig. 2b). By gel filtration chromatography on Superose 6 column ALA gave a single symmetrical peak eluted at 41.9 ml corresponding to molecular mass ~28 kD estimated by comparison with known protein standards (Fig. 2c). The absolute mass of the lectin was obtained as 27.3 kD from ESI-MS-Q-ToF mass analysis (Figure not shown).

Amino acid sequencing and glycan detection

The N-terminal amino acid sequence of the 16 kD protein band of ALA and its comparison with that of other lectins of the same family (Moraceae) is presented in Table 2. The N-terminal sequence of 30 amino acid residues of ALA showed close resemblance with *Maclura pomifera* agglutinin (MPA), *Artocarpus hirsuta* lectin (AHL) and Jack fruit lectin, jacalin, all belonging to the same family, Moraceae. They have the sequence homology with ALA at 15, 19–21, 24–27 and 29 residues.

Glycosylation pattern of the 16 kD subunit of ALA showed this subunit contained *N*-glycosidically linked "high mannose" or "hybrid"-type carbohydrate chains. 12 kD subunit did not react with the reagent indicating that this protein band might not be glycosylated (Fig. 3).

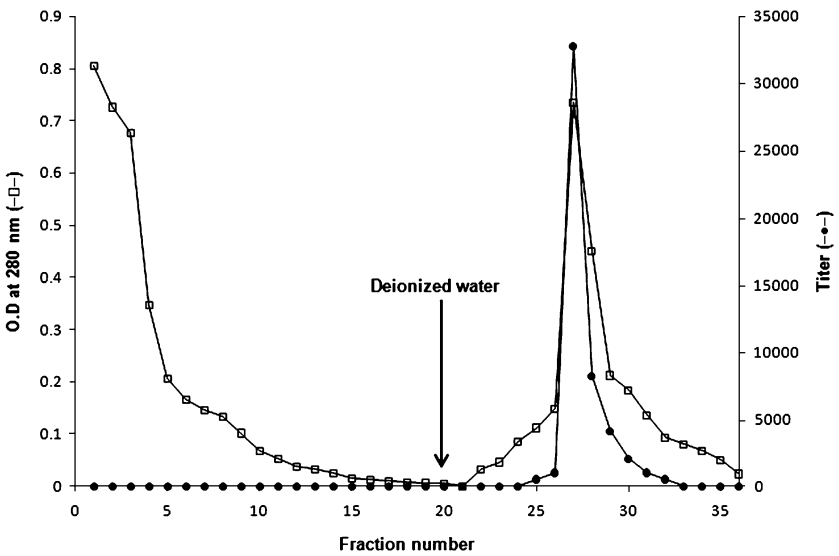

Fig. 1 Purification of *Artocarpus lakoocha* agglutinin (ALA). Affinity repulsion chromatographic profile of rivanol precipitated ALA on melibiose-agarose column (20 cm×1 cm). Protein elution was monitored spectrophotometrically at 280 nm, and activity was observed by hemagglutination of normal human B erythrocytes

Table 2 Comparison of the N terminal sequence of ALA (16 kD) with other lectin members of the same family

		1									10					
ALA		A	S	Q	T	I	T	V	G	P	W	G	G	P	G	
	G	N	G	W	**D**	**D**	**G**	S	Y	**T**	**G**	**I**	**R**	Q	**I**	E
	15															30
MPA	**G**	V	T	F	**D**	**D**	**G**	A	Y	**T**	**G**	**I**	**R**	E	**I**	
AHL	**G**	K	A	F	**D**	**D**	**G**	A	F	**T**	**G**	**I**	**R**	E	**I**	
JFL	**G**	K	A	F	**D**	**D**	**G**	A	F	**T**	**G**	**I**	**R**	E	**I**	

SPR analysis

SPR studies on the interaction of ALA with different glycoproteins and glycans showed changes in RUs (Fig. 4) and the association constants of ligands with ALA are given in Table 3. Among the glycoproteins tested for binding assay asialo BSM showed highest binding which was 340 times higher than BSM. There was not much difference in intensities of binding of asialo glycophorin and Tn glycophorin with ALA. Asialo fetuin showed moderate binding with ALA, which being 57 times higher than fetuin was almost analogous to asialo glycophorin. Among the glycans, Tn containing glycopeptide showed strongest binding followed by GlcNAc Tn. Methyl-α-GalNAc was found two times higher in binding potency than GalNAc and 260 times higher than Methyl-α-Gal. T disaccharide, Galβ1-3GalNAc showed least binding with ALA among the glycans tested except Methyl-α-Gal.

Biological properties

Mitogenic and antiproliferative activity

ALA was found to induce proliferation of human PBMC. The maximum proliferation was observed at a dose of 1 ng/ml of lectin (Fig. 5a). Thus, ALA could be designated as a very potent mitogen for human PBMC.

The antiproliferative activity of ALA on Jurkat cells was more intense (IC_{50} 13.15 ng/ml) than its effect on U937 cells (IC_{50} 25.14 ng/ml). K562 cells were less responsive than Jurkat and U937 cells since it did not reach 50% inhibition even at 40 ng/ml (Fig. 5b).

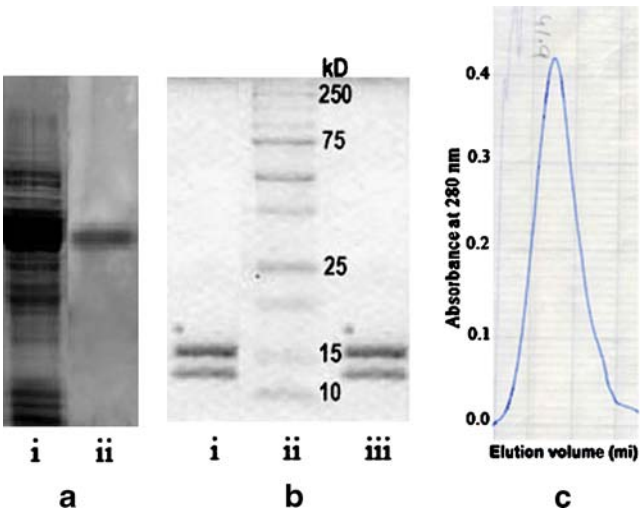

Fig. 2 **a** Non-denaturing polyacrylamide gel (10%) electrophoresis of ALA. *i* Crude extract (10 μg), *ii* Purified ALA (10 μg). **b** SDS-PAGE of ALA on 12% polyacrylamide gel *i* Denatured and non-reduced ALA (10 μg), *ii* Precision Plus Protein™ Standards from Bio-Rad, *iii* Denatured and reduced ALA (10 μg). ALA was denatured by 1% SDS and reduced by 2% 2-mercaptoethanol at 100°C for 5 min. The protein bands were stained with Coomassie brilliant blue G-250. **c** Gel filtration chromatography of ALA on FPLC by Superose 6, 10/300 GL column; the elution was made by 20 mM TBS at 4°C at a flow rate of 24 ml/h and monitored at 280 nm

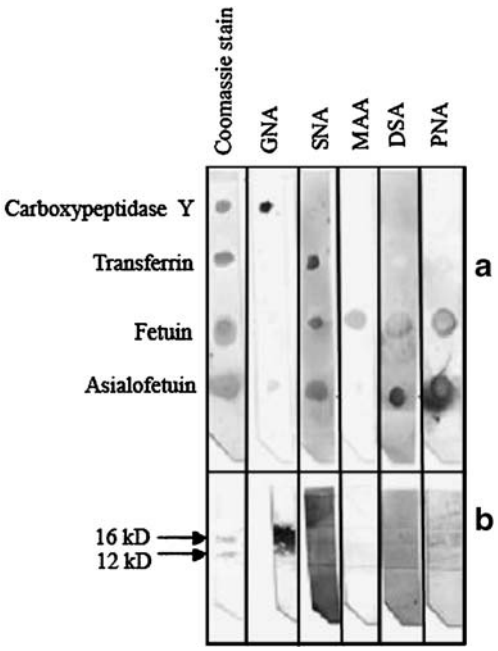

Fig. 3 Glycan detection in ALA subunit. **a** NCM showing specificity of the lectins present in the kit. **b** NCM demonstrating the reaction of ALA with the lectins in the kit. Positive reaction obtained with GNA for 16 kD band. GNA (*Galanthus nivalis* agglutinin), SNA (*Sambucus nigra* agglutinin), MAA (*Maackia amurensis* agglutinin), PNA (Peanut agglutinin), DSA (*Datura stramonium* agglutinin)

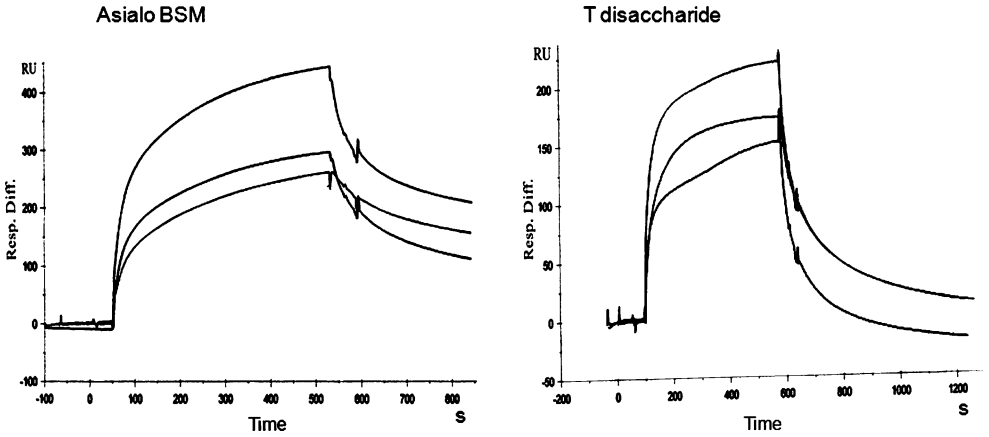

Fig. 4 Sensorgram of the interactions of immobilized ALA with asialo BSM and T-disaccharide by SPR. The surface of the CM5 sensor chip was activated with *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N*″-3(diethylaminopropyl) carbodimide (EDC; 1:1) at a flow rate of 5 μl/min for 30 min. ALA (200 μg/ml) in 10 mM Na-acetate buffer (pH 4.8) was immobilized onto the chip and the blocking was performed with 1.0 M ethanolamine hydrochloride (pH 8.5). The reference flow cell was prepared in an analogous manner without ALA. Various concentrations (5, 10 and 20 nM) of asialo BSM and T-disaccharide were injected onto ALA- immobilized sensor chip at a flow rate 5 μl/min for 8 min

Leukemic cell identification by QD-ALA conjugate

Cadmium sulphide quantum dot-conjugated ALA was applied as an efficient fluorescent probe to distinguish between leukemic and normal cells. The qualitative schematic representation is given in Fig. 6. In the present study QD-tagged ALA was synthesized in such a way that the incipient nanoparticles of cadmium sulphide were stabilized by ALA with its potential hydrogen bond forming functionalities and the efficient interactions with thiol (–SH) groups of cystein residues of the peptide fragment of lectin. The most effective QD-ALA conjugate was formed when Cd^{+2} and ALA were present in 1:2 molar ratio (378.12 μg ALA per μg of CdS nanoparticle) in the solution from which the QD-ALA nanoconjugates were generated. This molar ratio was maintained throughout this study. The formation of ALA stabilized QDs was monitored by photoluminescence spectra of the reaction mixture in which an excitation at 350 nm produced the characteristic photoluminescence of lectin capped CdS nanoparticle (Fig. 7a). The average size of the QD-ALA adduct was calculated from the TEM image (Fig. 7b) and was on the average 10 nm in diameter. EDX, obtained from the TEM experiment, proved the encapsulation of CdS nanoparticles in the lectin molecule (Fig. 7c). A color luminescence image that was obtained from the original water soluble QD-ALA conjugate was taken as guideline of the emission color of the QD fluorophore (Fig. 7d).

QD-ALA showed almost the same activity (titer 2^{15}= 32,768) as the purified ALA (titer 2^{17}=131,072). The fluorescent microscopic images revealed that QD-ALA conjugate could successfully and specifically bind to leukemic cell lines, which appeared as green fluorescent dots (Fig. 8a,b,c) whereas there was no such dot with normal lymphocytes (Fig. 8d). The absence of green fluorescence in case of normal lymphocyte is a direct proof of the target specific interaction of the QD-ALA with leukemic cells. It was also clear from the fluorescent images that the green fluorescence of the QD-ALA conjugate was localized only on the surface of the leukemic cells.

Table 3 Association constants for the binding of different glycoproteins and glycans to immobilized *A. lakoocha* agglutinin by SPR analysis

Glycan	K_a (M^{-1})
Asialo BSM	2.59×10^{8}
BSM	7.54×10^{5}
Asialo glycophorin	6.01×10^{6}
Tn glycophorin	5.37×10^{5}
Asialo fetuin	9.66×10^{6}
Fetuin	1.67×10^{5}
Galβ1, 3GalNAc	1.47×10^{5}
GlcNAc Tn	8.68×10^{5}
GalNAc	2.76×10^{5}
Tn containing glycopeptide	9.12×10^{5}
Me α GalNAc	6.10×10^{5}
Me α Gal	2.39×10^{3}

Discussion

A. lakoocha agglutinin purified by successive precipitation with ammonium sulphate and rivanol followed by affinity chromatography on melibiose-agarose column is a heterodimer comprising of two subunits 16 and 12 kD respec-

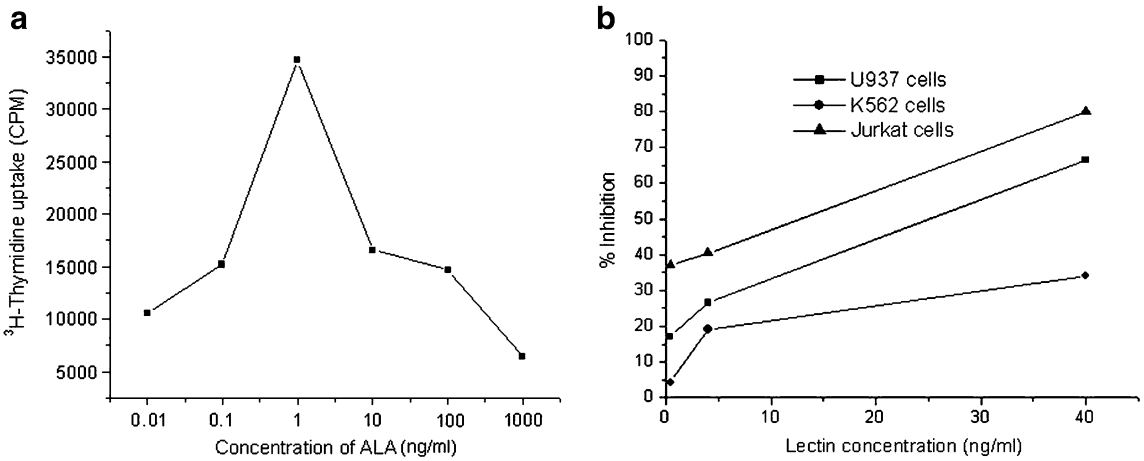

Fig. 5 **a** Mitogenic activity of ALA toward human PBMC observed as the count per min (CPM) of $[H]^3$ thymidine uptake. **b** *In vitro* inhibitory effect of ALA on proliferation of leukemic cell lines. IC_{50} on Jurkat cells, 13.15 ng/ml; IC_{50} on U937 cells, 25.14 ng/ml and K562 cells did not reach 50% inhibition even at 40 ng/ml

tively and of molecular mass ~28 kD. Heterodimeric nature of subunits of molecular weight 19 and 22 kD respectively was observed in *Artocarpus altilis* lectin, a member of Moraceae family [30]. The molecular weight of jacalin was reported to be 62 kD consisting of two non identical subunits of molecular weights 13 and 18 kD respectively [31]. Like jacalin, AHL and MPA, ALA showed strongest affinity for binding to Me-α-Gal/GalNAc.

The N-terminal sequence of ALA showed close homology to jacalin, AHL, MPA all belonging to Moraceae family. Thus ALA can easily be called a jacalin like lectin. Structural pattern of glycoform of ALA indicated mannose terminally linked to the 16 kD subunit and that the 12 kD subunit was non-glycosylated.

In the SPR studies ALA showed high binding affinity with glycoproteins in the order of asialo BSM > asialo fetuin ≅ asialo glycophorin > Tn glycopeptide ≅ Tn glycophorin ≅ GlcNAc Tn ≅ Me-α-GalNAc. BSM contains over 53% sialyl Tn and over 22% GlcNAc Tn (GlcNAcβ1→3Tn) as major carbohydrate side chain. The next abundant lectin determinant (lectin binding saccharide) in BSM is sialyl Tα and GlcNAc β1,6Tα. Complete removal of sialic acid by neuraminidase resulted in high abundance of Tn structure (Fig. 9) [32]. Glycophorin which is the human erythrocyte glycoprotein contains fifteen O-linked sialyl Tα structure. Disialylation of glycophorin resulted in asialoglycophorin which upon ß-galactosidase treatment produces Tn glycophorin (Fig. 9) [33].

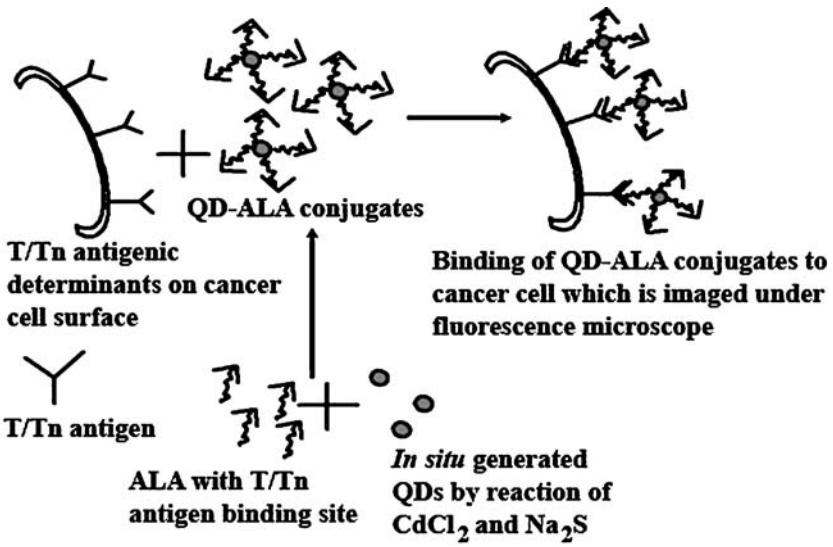

Fig. 6 Qualitative schematic presentation of QD-ALA conjugate formation and its successive binding with leukemic cells. CdS nanocrystals are generated *in situ* by the reaction of $CdCl_2$ and Na_2S in presence of ALA, which contains T/Tn antigen binding sites. These fluorescent QD-ALA conjugates on incubation with leukemic cell lines having T/Tn antigenic determinants exposed on their cell surface, binds to the cells which are then imaged under fluorescence microscope

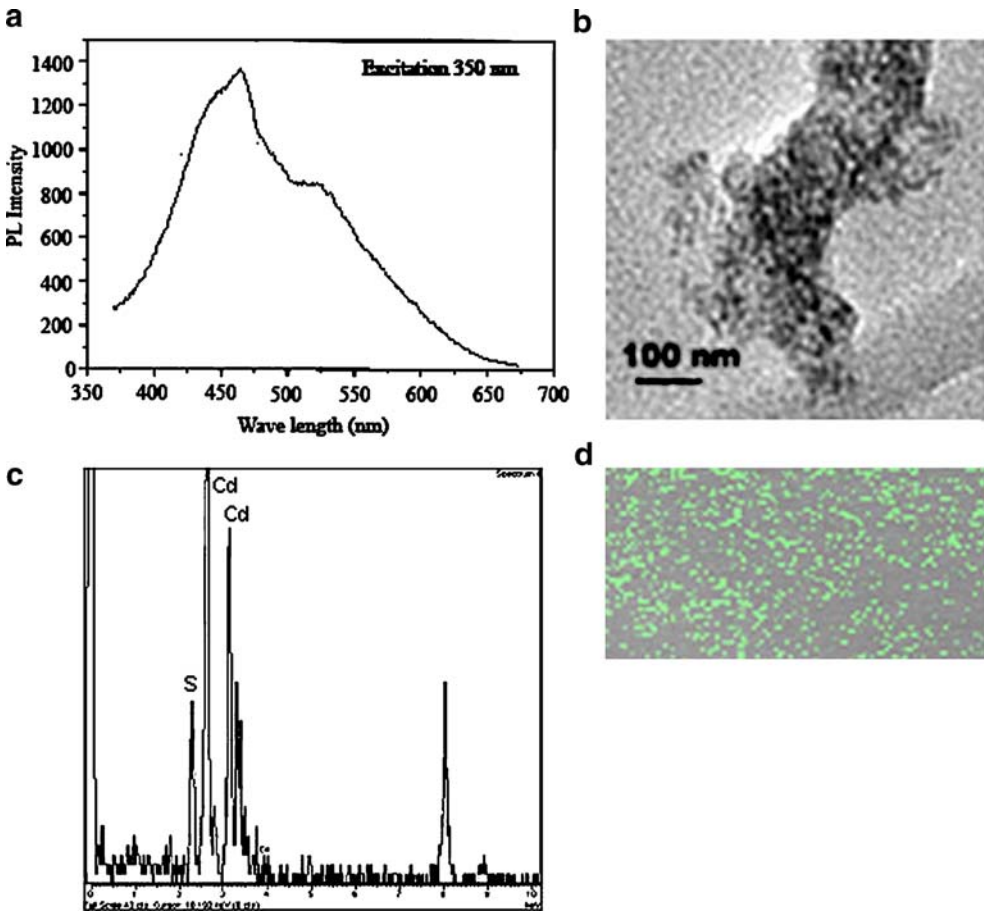

Fig. 7 **a** Photoluminescence spectra of QD-ALA conjugate. **b** TEM image of QD-ALA conjugate. **c** Electron dispersive X ray of QD. **d** Color luminescence images of QD-ALA

Fetuin contains six glycan chains per molecule; three of them are O-linked sialyl Tα attached to Ser or Thr residues of the protein core and the other three are N-linked sialyl bi- or tri antennary glycans of ratio 2:1. Thus asialo fetuin (Fig. 9) contains two lectin determinants Tα and II, a precursor of blood group antigens present at the non-reducing end of the carbohydrate chains of N-glycans [34]. ALA binds specifically to tumor associated carbohydrate antigens, GalNAcα1→Ser/Thr(Tn) and Galβ1→3GalNAcα1→Ser/Thr (Tα). SPR analysis confirmed our previous results of ELLSA which showed the binding affinity of ALA for Tn/Tα containing glycans in the order of asialo HSM > asialo

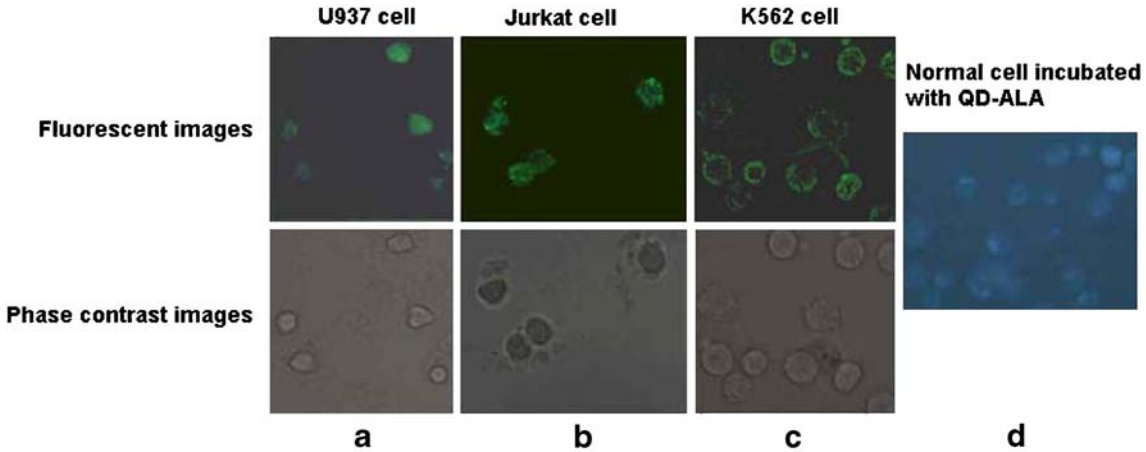

Fig. 8 Fluorescent and phase contrast images of QD-ALA bound to **a** U 937; **b** Jurkat; **c** K562 cell lines; **d** Normal lymphocytes incubated with QD-ALA did not fluoresce

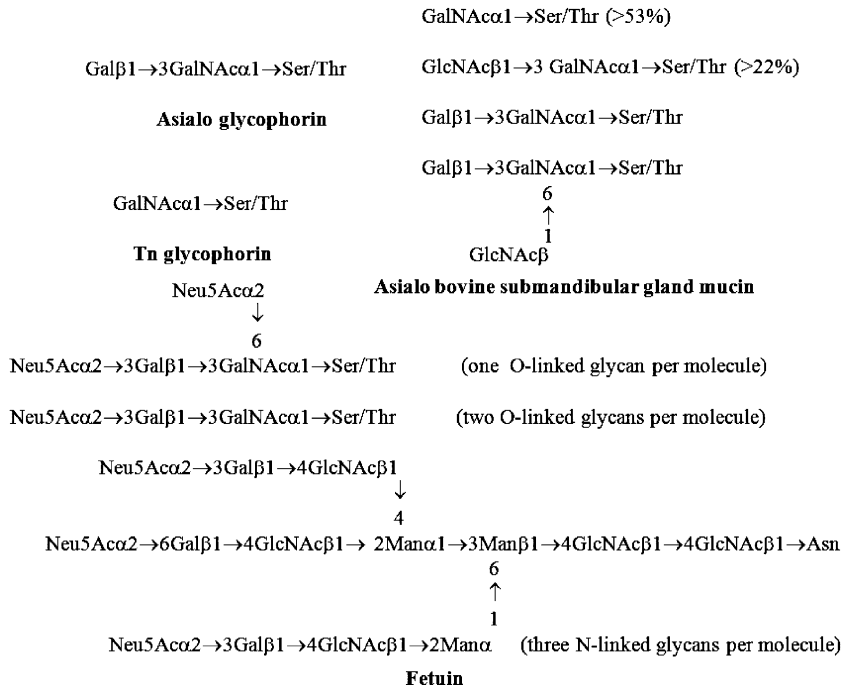

Fig. 9 Glycan structures of mammalian glycoproteins

OSM=asialo BSM > asialo PSM ≅ Tn glycophorin > asialo glycophorin ≅ Tn glycopeptides > monomeric Tn > GalNAc [15]. Although the binding studies of ALA with Tn/Tα glycotopes have been performed by different methods yet the binding order is almost the same. The enhanced specificity of ALA for Tn/Tα cluster suggests the importance of glycotopes polyvalency during carbohydrate-receptor interaction. Thus, ALA is considered a valuable reagent for analysis of progression of cancer since there is a direct link between carcinoma aggressiveness and the density of these antigens, including extent of tissue spread and vessel invasion [35, 36].

ALA was endowed with a potent mitogenicity effect toward human PBMC. It also exhibited a potent inhibitory effect on the proliferation of leukemic cell lines. This finding is in concurrence with the previous reports on many other lectins [28, 37–40]. Lectins have been used for gastrointestinal cell targeting. Tomato lectin and jacalin retained their antitumor activity through the gastrointestinal tract [41].

The novelty and importance of newly developed CdS quantum dot-ALA nanoconjugate lies in differentiating between leukemic cells and normal lymphocytes since the lectin binding sites are cryptic in normal cells but are exposed in leukemic cells. The application of this nanoconjugate is based on the ability of its lectin part to recognize the specific target glycotopes on the cancer cell surface and to visualize this binding by novel fluorescence property of the QD. Introduction of CdS (QD) in the lectin based nanoconjugate also enhances its efficiency as compared to the conventional organic fluorescent dye tagged lectin sensors. Due to its very high surface/charge ratio more than one lectin molecule can interact with a single nanoparticle. Thus the nanoconjugate with multi-lectin molecule becomes more efficient in interacting with the cell surface glycoproteins rather than a conventional one to one lectin molecule-fluorescent dye conjugates. No green fluorescence was observed in normal lymphocytes. The TEM result justified the conjugation of ALA molecule with a single quantum dot nanocrystal, rather than the conjugation of one or more quantum dots with quaternary structure of ALA. This completely nullified any potential effect of QD on biological activity of ALA. Retention of hemagglutination activity of QD-ALA conjugate demonstrated it clearly. In this study the technique used was based on *in situ* generation of QD in presence of lectin leading to the formation of lectin capped QD to minimize the lectin-lectin cross linking.

In conclusion it can be stated that QD-ALA nanoconjugate is an efficient fluorescent marker for identification of several leukemic cell lines that gives rise to high quality images and possesses higher stability against photobleaching.

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