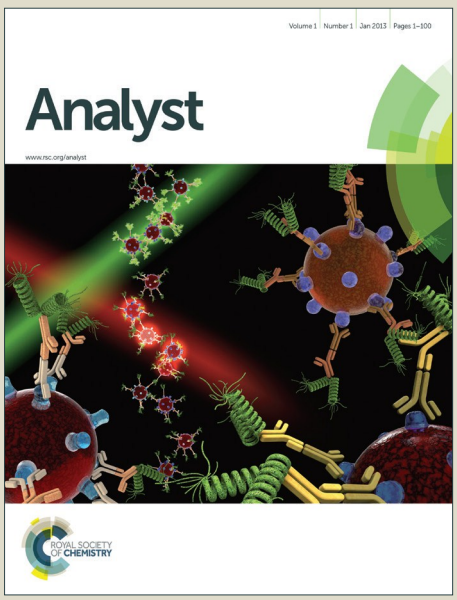
Analyst

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

Visual detection of serum asialohaptoglobin by plasmonic sandwich ELLSA- a new platform for cirrhosis diagnosis

Partha Pratim Bose,[a,b*] Gautam Mandal,[c] Dharmendra Kumar, [a] Ajay Duseja,[d] Bishnu Pada Chatterjee.[c]

[a]Department of Biotechnology
National Institute of Pharmaceutical Education and Research
Hajipur 844102, India
[b]Current address: Division of Molecular medicine, Bose Institute, Kolkata 700054
[c]Department of Natural Science
West Bengal University of Technology,
Kolkata700064, India
[d]Department of Hepatology,
Post Graduate Institute of Medical Education and Research
Chandigarh 160012, India
[*]E-mail for correspondence (PPB): get.ppb@gmail.com; ppb.mm@jcbose.ac.in

Abstract Cirrhotic condition of liver has long been attributed to the preface to liver cancer. The desialylation status of the serum acute phase protein, haptoglobin has been introduced as a new diagnostic analyte for liver cirrhosis. The reliability of this new diagnostic molecule has been evaluated with 30 liver cirrhosis patients having history of earlier viral hepatitis C (HCV-LC). A novel enzyme linked lectinosorbent assay has been developed coupled with plasmon mechanism of gold nanoparticle aggregation as the colorimetric read out which can visually distinguish the cirrhotic liver patients from the normal healthy and hepatitis C controls. The assay can be useful for rapid point-of-care detection, which even untrained person can execute without specialized instrument. This method employs *Sambucus nigra* agglutinin (SNA) to detect the extent of α-2,6 sialylation of serum haptoglobin, the new diagnostic molecule for liver cirrhosis.

Introduction

Malignant transformation significantly alters the N-glycan structures of cells. This occurs due to altered expression of various glycosyltransferases that results in increased branching of N-glycans in many cancers.[1-3] These glycoproteins with altered oligosaccharide structures are released from tumor cells to bloodstream either via classical secretion mechanism or by enhanced proteinase activity of various matrix metalloprotinases (MMP). These aberrant secretomes can act as serological biomarkers of various cancers.[4] However, intrinsic complexity of glycan structures of the marker glycoproteins makes their analysis difficult. Mass spectrometry and tandem nanoflow LC-MS can quantify the monosaccharide composition as well as their types of linkage, which are crucial for the new diagnostic development.[5-7]

Hepatocellular carcinoma (HCC) is the fifth most common cancer in men and the seventh in women in the world with more than 748,000 new cases being diagnosed each year, and has become one of the leading causes of cancer related death worldwide.[8,9] Despite the virulence of the disease it is most often diagnosed at an advanced stage leaving a little chance for therapeutic intervention. Most often HCC occurs in cirrhotic livers following chronic infection with hepatitis B virus (HBV) or hepatitis C virus (HCV) and reports suggest that 60%-80% of HCCs are preceded by cirrhosis.[10-12] Indeed, liver cirrhosis (LC) is a notable risk factor for the development of HCC. Therefore, there is an urgent need to diagnose LC for better clinical management of HCC. Widely used imaging techniques, such as ultrasonography, computed tomography scanning, and magnetic resonance imaging cannot distinguish benign cirrhotic macronodules from HCC.[13,14] Till date in the diagnosis and treatment follow-up of LC and HCC, serum α-fetoprotein (AFP) has been considered as the only serological marker.[15,16] However, the sensitivity and specificity of AFP for its wide variation is questionable.[17] Therefore, newer specific and sensitive serological markers are needed for the early detection of LC. Desialylation of glycoproteins such as α-acid glycoprotein (AGP), Transferrin (Tf) etc in the hepatocyte membrane and serum caused by the down regulation of liver Galβ1,4GlcNAc α2,6-sialyltransferase has long been attributed to be the crucial feature in LC and alcohol induced liver damage which are mostly considered as the prelude to HCC.[18,19] Serum concentration of haptoglobin (Hp) and its fucosylation has recently been shown to correlate with the transformation of LC to HCC.[20-22] In our current endeavor we have correlated serum

concentration of asialo-Hp with the occurrence of LC in Indian patients. Here we have introduced a cheap, fast, point-of-care and high throughput visual assay platform for specific identification and determination of asialo-Hp to monitor the cases of LC. We used *Sambucus nigra* agglutinin (SNA) having specificity for NeuAc (α2-6) Gal/GalNAc residue to determine sialylation level of Hp in normal subjects and enhanced desialylation in LC patients in a novel sandwich Enzyme Linked Lectinosorbent Assay (ELLSA) for this purpose.[23-24] We have harnessed gold nanoparticle (GNP) based plasmonic technique in this new sandwich ELLSA platform to achieve high sensitivity and visual detection by distinct change in color. The visual color change from red to blue is due to the aggregation of GNPs that can be triggered to different extent due to variation of the concentration of consumed hydrogen peroxide (H_2O_2) added as reducing agent. The color change can directly be correlated to the extent of sialylation on Hp by a coupled peroxidase mechanism.[25-26]

Results

Serum Asialo-Hp levels in HCV-LC patients

To choose the lectin and to validate the detection of α 2,6-sialic acid linkage in Hp, lectin-blot analysis was performed with five lectins having different carbohydrate specificities: *Sambucus nigra* agglutinin (SNA; specificity: Neu5Ac α2,6 Gal), wheat germ agglutinin (WGA; specificity: GlcNAc, GlcNAc β1-4GlcNAc, Neu5Ac), concanavalin A (ConA; specificity: Glc, Man, GlcNAc, branched α-mannosidic structures; hybrid type and biantennary complex type N-Glycans), (*Galanthus nivalis* agglutinin (GNA); specificity: α 1-3 and α 1-6 linked high mannose structures), (Soybean agglutinin (SBA); specificity: N-acetyl-D-galactosamine, Gal). Commercial Hp (human) showed much higher reactivity with SNA than that with WGA and ConA but no reactivity was observed with SBA and GNA (Fig. 1A). This established the reactivity of SNA with α-2,6 sialic acid linkage on the commercial Hp. To assess the extent of desialylation of Hp in the sera of hepatitis C induced liver cirrhosis (HCV-LC) patients as well as normal subjects, the concentration of Hp was measured by ELISA with anti-Hp antibody. The range for control and HCV-LC were found to be 435±108 μg/ml versus 533±109 μg/ml (ESI Fig. SI).

To determine the binding of SNA, if any, with mAb-Hp (monoclonal anti-Hp), we went on to compare the ELISA results with periodate treated and non-treated mAb-

Hp.[27] The binding of SNA with Hp was increased proportionally with increasing concentration of both periodate treated and non-treated mAb-Hp with no significant difference. On the other hand no appreciable increase in binding of SNA was observed when the amounts of both treated and non-treated antibody were increased in the absence of Hp (Fig. 1B). Thus, no substantial background binding of SNA with mAb-Hp was observed that would downplay the sensitivity of detection of α-2,6 sialic acid linkage on Hp by the reactivity of SNA.

Next we went on to study the sialylation status of Hp directly from clinical samples (sera) by lectin blot analysis with SNA. Sialylation status of serum Hp by lectin blotting showed significant reduction of binding in HCV-LC patient groups (p <0.001) as compared to healthy and HCV controls (Fig 1C). Following the blotting experiment, densitometric analysis of blots by Image J software also revealed the substantial difference in lectin (SNA) activity of serum Hp between liver cirrhosis patient and control groups (Fig 1D). Next, we studied the same SNA activity of the serum Hp i.e. the extent of α-2,6 sialic acid linkage on Hp by ELISA method.

For our subsequent sandwich ELISA for measuring the extent of desialylation of Hp by subsequent use of anti-Hp antibody and biotinylated SNA, we diluted the sera to maintain the Hp added per well to an amount of 1μg, both for patients groups and control groups and this allowed us to compare the sialylation status of Hp for patients and that of controls directly from the OD values of the corresponding wells. We have included two types of controls in our study, normal healthy controls (n = 20) and patients suffering from hepatitis C virus infection (HCV-controls) (n = 10). We took these subjects under a common group ‘control’ because liver cirrhosis does not have a very severe symptom and till date, only by hematologic tests it is not at all possible to distinguish between viral hepatitis C and liver cirrhosis. Therefore, our ultimate aim had been to distinguish the liver cirrhosis patients from this common control group. The diagnostic criteria for selection of patients and controls were according to standard clinical guidelines. Several hematological tests such as elevated serum bilirubin-conjugated, elevated AST, ALT (AST/ALT ratio > 1), ALP, AFP, low serum albumin, deranged prothrombin time had been included (Table 1). In our study we also included standard noninvasive imaging technique (Ultrasound imaging) as one of the diagnosis criteria for our subject selection and the extent of liver damage in cirrhotic patients was assessed by this radiological test (Table 1). To validate our

results from lectin blot analysis with clinical samples we studied the extent of sialylation of serum Hp by ELISA and compared them with the available clinical assessments (as in Table 1) for each of the individuals. The extent of Hp-sialylation was found to be significantly less ($p<0.0001$) in HCV-LC patients groups (0.248 ± 0.034) than control groups (0.315 ± 0.022 for healthy controls and 0.286 ± 0.022 for HCV controls) in the ELISA (Fig. 1D).

Development of plasmonic ELLSA

For sensitive and fast visual determination of the sialylation status of Hp in LC we developed a plasmonic Enzyme linked lectinosorbent assay (ELLSA). In this assay (Fig. 2) we introduced the gold nanoparticle (GNP) as colorimetric index, which enabled the assay platform to be readable by naked eye with excellent sensitivity due to the plasmon characteristics of GNP.[25-26] The target acute phase protein Hp was captured either from the solution of commercial Hp at different concentrations (for optimization) or from patient's sera (for real sample analysis) by anti-Hp antibody on the 96-wells polystyrene plate. The degree of sialylation of arrested Hp molecules was assessed by the lectin SNA. In conventional ELISA colorimetric read out is carried by using enzyme based chromogenic reactions where concentration differences of analytes are estimated by plate reader.

To perform naked eye based colorimetric detection, we employed plasmonic GNP based colorimetric read out technique for understanding the subtle change of sialylation profile in Hp. Following figure 2 in each well of this assay platform, equal amount of gold (III) solution was added which was subsequently reduced to generate the GNPs in presence of added hydrogen peroxide (H_2O_2) as reducing agent. The concentration of left over H_2O_2 was varied with the extent of sialic acid present on bound Hp to anti-Hp antibody in each microtiter wells because antibiotin tagged horseradish peroxidase (HRP) was used after the addition of tracer molecule, biotinylated SNA. Obviously, biotinylated SNA got bound according to the α-2,6 sialic acid concentration on Hp in each well, which in turn controlled the concentration of subsequently, added HRP in each wells. Thus due to the difference in concentration of sialic acid on Hp in different wells, the concentration of peroxide cleaving HRP got varied which gave rise to difference in concentration of left-over H_2O_2 (Fig. 2). More the sialylation, more amount biotinylated SNA will bind to Hp as

well as more amount antibiotin-HRP conjugate would bind to SNA in the system and thus more amount of peroxide would have been cleaved. This low concentration of available peroxide for reduction of gold (III) affected the formation kinetics and thus the aggregation propensity of the GNP, which appeared as blue colored nonspherical, aggregated particles.

In agreeing the principle we first went on to optimize the gold (III) and H_2O_2 concentration with respect to serially diluted commercial Hp to produce tonality difference distinguishable by naked eye, which will help us to determine the sialylation status of Hp visually. We optimized the color change of GNPs grown under different concentrations of added peroxide with respect to same amount of gold (III) and their spectral characterization was also carried out (Fig. 3A). It has been found that up to a concentration of 150 μM of H_2O_2 in the wells the 0.2 mM of added Au (III) will produce red GNP dispersible in the medium with a steady absorbance maximum around 550 nm and when the concentration of H_2O_2 decreases below 150 μM, the blue GNPs were formed. At 120 μM purple transition color was observed (Fig. 3A). The drop of absorbance at 550 nm is for blue particles showed a linear trend (Fig. 3B), which could make the basis of the assay platform and estimate the extent of desialylation of Hp in control as well as patient's sera. The shift in plasmon band towards higher wave length due to the formation of aggregated blue GNPs from discrete red GNPs was depicted in Fig. 3C. After optimization of concentration of hydrogen peroxide with respect to the specific amount of gold (III), we validated the plasmonic phenomena in a ELLSA based detection platform where α-2,6-sialic acid contain of the commercial Hp was used as a measurable analyte by the specific SNA interaction as the concentration of added Hp was varied well to well (Fig. 3D-E). During the optimization steps it was found that MES buffer (2-(N-morpholino) ethanesulphonic acid) which was used for dissolving the gold (III) and also for diluting peroxide solution, showed some reducing property and the gold (III) solution became faintly blue upon keeping for more than 20 minutes. Therefore, to avoid any false detection we performed the assays with freshly prepared gold (III) and solutions. To validate the naked eye detection of Hp-sialylation level by red-blue plasmonic color in terms of experimentally determined quantity, we studied the difference in absorbance at 550 nm with respect to the increasing concentrations of Hp and a control protein, bovine serum albumin (BSA) and the difference in the absorption at

550 nm between the same concentrations of Hp and BSA, '-ΔA550' (where -ΔA550 = -(A550 for Hp - A550 for BSA)) were plotted against the concentration of proteins. Figure 3D-E demonstrated that with increase in concentration of Hp, red colored GNP were formed with a linear increase in the value of '-ΔA550'. In our optimized experimental condition (with respect to concentrations of added SNA, H_2O_2 and Au(III) solution) a visibly distinct color change from red to blue was observed around 1 mg/dl of Hp concentration and the value of '-ΔA550' did not change with further increase in Hp concentration in our experimental condition (Fig. 3E). This concentration of Hp was maintained for all the subsequent assays with clinical samples as it was envisaged that in this specific amount of serum Hp in each well, color change from blue to red occured due to higher de-silylation which was the characteristic of liver cirrhosis with an concomitant decrease in the value of '-ΔA550' with the extent of desilylation of Hp. It was also evident that the decrease in the values of '-ΔA550' for red colored wells were linear with respect to the decrease in Hp added in those wells and thus the values of '-ΔA550' reflected the linear decrease in over all Hp-sialylation which could be related to the progress of cirrhotic condition of liver during the real sample analysis.

The transmission electron microscopic images of red and blue GNPs in Fig. 3F and G illustrated the morphological differences and aggregation state of GNPs as nonaggregated red (quasi-spherical) and aggregated blue (irregular) particles. The optimization amount of gold (III) and H_2O_2 in the wells to achieve visibly convincing color change from red to blue with decreasing amount of reducing agent (H_2O_2) was done as demonstrated in ESI Fig. SII.

Visual detection of HCV-LC by plasmonic ELLSA

After establishing the prevalence of desialylated Hp in cirrhosis patients sera to be a potential diagnostic analyte for liver cirrhosis by classical ELISA method and optimization of plasmonic visual detection platform, we tried to employ the GNP based plasmonic phenomena in a novel lectin based assay (ELLSA) for visual detection of the Hp sialylation in clinical samples. Fig. 4A demonstrated that all the 8 patients of HCV-LC can be visually distinguished from the control groups comprising of 2 healthy and 2 HCV-controls just by color of the wells, for patient's group the color was red and for control groups bluish purple made the discrimination prominent.

Those patients and controls were randomly selected from the same sets of patients and controls as previously tested by classical ELISA technique (Fig. 1E). Values of '-ΔA550' were determined for each subjects (Fig. 4B-C) and that were corroborated with the fact established in Fig. 3D-E that with the substantial desilaylation of Hp in liver cirrhosis there would be a visible color change from blue to red which would be associated with an significant decrease in the values of '-ΔA550' in patients group than the controls (Fig. 4B-C).

Discussion

The classical ELISA results with patients sera signified that the level of α-2,6 sialylation of Hp was less in patients group than healthy control, commensurating with the crucial previous observations in liver cirrhosis: the increased desialylation either by enhancement of sialidase activity or by decrease in sialyltransferase activity.[28] Thus our ELISA result convincingly established the correlation between Hp-sialylation status and the occurrence of LC.

Next in our endeavor we established a visual assay platform based on plasmon characteristics of GNP to assess the desialylation status of Hp from colorimetric assay. Color of GNP is the reporter of its aggregation state and extent of aggregation can vary with concentration of reducing agent present in the medium. In case of less Hp-sialylation and consequently, according to the design of assay, the concentration of HRP was also less. Therefore, the GNP was produced under higher concentration of peroxide, their formation kinetics was fast and the quasi-spherical homogeneous GNPs were formed which produced distinct red coloration in the solution of the well. Whereas, in case of more Hp-sialylation, HRP concentration was also increased and thus aggregated, nonspherical, blue GNP was formed. This change in color from red to blue depending on the extent of sialylaion of Hp was starkly distinguishable by naked eye. We had extended the plasmonic visual ELLSA platform to detect the HCV-LC cases distinctly from the healthy normal and HCV patients serologically. It was found that the novel plasmonic ELLSA was successful in distinguishing HCV-LC cases by the appearance of red color as compared to purple blue in normal healthy controls. To substantiate the visual detection of the extent of Hp-silylation, an experimental quantity '-ΔA550' was determined in correlation with the visual color change with the change in Hp-sialylation level. The value of '-ΔA550' dropped

significantly for the HCV-LC patients as compared to the controls. Thus drop in the '-ΔA550' value signified higher Hp-desialylation in cirrhotic liver condition and that could be visually detected by 'red' color in our visual assay in comparison with 'blue' color in all non-cirrhotic controls.

This new assay could prove to be beneficial for easy and fast detection and monitoring of cirrhosis cases by naked eye. By seeing the color change one could be able to conclude the desialylation status of serum Hp.

Experimental Section

UV-Visible spectra

All absorbance measurements were carried out in UV/Vis spectrophotometer (Shimadzu 1800) using quartz cuvette of 1 cm path length. The absorption spectrum was measured from 700 to 450 nm against PBS buffer

Determination of lectin reactivity of commercial Hp

For lectin blot, equal amount of Hp (10 µg, Sigma) was subjected to 10 % SDS-PAGE in each lane. The protein bands were electroblotted onto nitrocellulose membrane. The membranes were blocked with 5% BSA (w/v) in PBS containing 0.1% Tween -20 (PBST) for 1 h at room temperature, washed and were separately incubated with the following biotinylated lectins: *Sambucus nigra* agglutinin (SNA), wheat germ agglutinin (WGA), concanavalin A (ConA), soybean agglutinin (SBA) and *Galanthus nivalis* agglutinin (GNA), each diluted to 1:1000 using blocking buffer. After washing with PBST the membrane was incubated with HRP conjugated streptavidin (dilution 1:10,000) in PBST. Reactive protein bands were visualized by addition of diaminobenzidine and 0.01% H_2O_2 in sodium acetate buffer (pH 5).

Subjects and ethics statement

Serum samples from 30 hepatitis C virus induced liver cirrhosis (HCV-LC) patients (study group) were collected from out patient clinic of Hepatology, Post Graduate Institute of Medical Education and Research, (PGIMER), Chandigarh, India. The sera from 20 age and sex-matched healthy individuals and 10 patients sufferening from hepatitis C (HCV) were taken as controls. All subjects were diagnosed by serological-

radiaological detection according to the standard mediacl practice (Table 1) and were recruited in this study with their informed consent. Ethical committee of the PGIMER approved this study (Micro/2010/1092/3rd Mach 2010).

Determination of sialylation level of Hp in patient's sera and normal healthy controls by classical ELISA

The sialylation level of haptoglobin was performed by ELISA using sialic acid specific lectin, *Sambucus nigra* agglutinin (SNA, specificity: NeuAcα2-6 Gal). The wells of microtiter plate were coated with 100 µl (2 µg/well) of monoclonal anti-haptoglobin (mAb-Hp, Sigma) in bicarbonate buffer (0.01 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6). The plates were kept at 4°C for 24 h, washed with 100 µl 0.01 M PBS, pH 7.4, containing 0.05% Tween-20 and incubated with 100 µl of PBS containing 1% BSA at 37 ^{0}C for 1 h. To each well, 100 µl of diluted sera of HCV-LC patients and control groups were added (Hp concentration was adjusted to 100 mg/dl). The plates were incubated at 25°C for 1 h and then 100 µl of biotinylated SNA (1:1000 in blocking buffer) was added. On further incubation for 1 h and washing (three times as previously), 100 µl of streptavidin-HRP conjugate (1:10,000 in blocking buffer) were added and incubated at room temperature for 1 h. After that 0.1% *O*-phenylenediamine dihydrochloride (OPD) (100 µl) and 0.05% H_2O_2 in 0.05 M citrate phosphate buffer (pH 5.0) were added to each well. The plate was left for 30 min at room temperature. The absorbance of each well was measured at 490 nm in an ELISA Reader. All experiments were done in triplicate.

Determination of lectin reactivity on monoclonal Hp by ELISA

Deglycosylated monoclonal Hp antibody (mAb-Hp) was prepared by sodium periodate oxidation. Monoclonal antihuman Hp was treated with 50 mM sodium periodate in 50 mM sodium acetate, pH 4.0 followed by incubation for 2 h in the dark at 4 ºC. Next, the reaction mixture was dialyzed into PBS (pH 7.4) overnight with three changes of buffer prior to use in ELISA.

Each well of a 96 well microtiter plate (NUNC) was coated with different concentration of treated as well as non-treated mAb-Hp (1, 2, 3, 4 and 5 µg/well) in buffer (0.01 M Na_2CO_3 and 0.035 M $NaHCO_3$, pH 9.6) and kept at 4 °C for 24 h. The rest of the procedure was same as in classical ELISA above.

In another experiment the binding of lectin with treated and non-treated mAb-Hp were studied in the absence of Hp. Wells were coated with different concentration of both treated and non-treated mAb-Hp similarly as before. Here after blocking and washing with PBST, biotinylated SNA was added to the wells without addition of commercial Hp. Then antibiotin–HRP conjugate was added to the wells followed by addition of substrate and rest of the procedure was same as mentioned above.

Lectin blot analysis with sera from HCV-LC patients and controls

The sialylation level was monitored in both control and HCV-LC patient groups by lectin blotting using SNA. Equal amount of albumin and IgG depleted proteins (20 µg), from pooled sera (two groups, HCV-LC and control with 10 samples each) of both groups were separated on 10% SDS-PAGE. After the separation, proteins were electroblotted onto nitrocellulose (NC) membrane with a constant current 80 mA for 2 h at room temperature. The rest procedure was done as described in the above. The intensity levels of bands were calculated using Image J software. The statistical analysis was performed by One-Way ANOVA test ($p<0.05$).

Plasmonic ELLSA for determination of sialylation of Hp

Different concentrations of commercial Hp and BSA (0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 mg/dl) were added to the wells of ELISA plate and kept for 1 h at room temperature. After washing with 100 µl 0.01 M PBS, pH 7.4, containing 0.05% Tween-20, 100 ul of 150 µM H_2O_2 (in MES buffer, 2-(N-morpholino) ethanesulphonic acid, pH 6.5) and freshly prepared 100 ul of 0.2 mM gold solution ($HAuCl_4$) in MES buffer were added to each well. The color change of the solution was observed after 15 min. Plasmonic ELLSA was performed with patients' samples as done above in classical ELISA following the addition of gold solution in MES buffer.

Characterization of aggregation state of GNP from assay by Transmission Electron Microscopy (TEM)

Aggregation state of red and blue GNPs obtained after the assay was determined by TEM analysis. Briefly, 20 µL of the red/blue GNP solution from the wells of the ELISA plate were dropped on carbon coated copper grid (300 mesh). Then the grid

containing the drop of sample solution was dried under vacuum. All the TEM measurements were performed in JEOL 2010 under an accelerating voltage 100 kV.

Conclusions

With newer glycoprotein analytes being included in modern diagnostics it has been an urgent need to compare the carbohydrate portion of the glycoproteins where there has been a shift in paradigm that the subtle changes in carbohydrate composition can be a more specific and sensitive biomarker than the overall concentration change of that corresponding glycoproteins. Hepatic clearance of serum glycoproteins are characterized by the hydrolysis of terminal sialic acid exposing the galactose to be subsequently captured by surface asialogycoprotein receptor of liver cells to remove them from circulation. Liver cirrhosis being the intermediate stage in between HCV and HCC demonstrates subtle difference in the mentioned asialylation of glycoproteins. For the first time to our current endeavor we have demonstrated the clinical correlation between the increase in serum concentration of desialylation of Hp and the clinical manifestation of the HCV-LC from HCV. Liver biopsy has been accepted as gold standard method in the determination of degenarative liver condition like in cirrhotic liver, however, a recent cohort study with liver cirrhosis patients demonstrated that even after liver biopsy, cirrhosis remained underdocumented and underdiagnosed in many cases.[29] Over and above, this highly invasive study can not be adopted as monitoring procedure or as routine investigation as existing literature indiactetd a substantial mortality following liver biopsy because of fatal complications such as pneumothorax, perforation of other organs, sepsis along with heavy bleeding.[30] A recent multi-cohort mathematical model of Hepatitis C predicted an alarming increase in the proportion of cirrhosis from 25% in 2010 to 45% in 2030 worldwide.[31] Therefore, a convincing, fast, easy serological test requiring no specialized technical support is an urgent need for the diagnosis and management of cirrhosis in time of this liver disease pandemic. In this current study we have established that increase in serum asialo-Hp can be employed as the marker to determine the progress of HCV to HCV-LC, which reflects the damage of the surface asialoglycoprotein receptors on the liver cells in cirrhotic condition. We have demonstrated a new non-MALDI, non-chromatographic assay platform for estimation of sialylation of Hp directly from whole serum by a new GNP-plasmon based visual

detection assay for easy, cheap and fast detection of serum Hp-desialylation status which would prove to be effective for visual detection of liver cirrhosis and serological monitoring of the same. This assay can determine the level of asialo-Hp and thus can distinctively monitor the clinical cases of HCV and HCV-LC only by visually distinct color change from red to blue and can be very useful in resource stringent countries. Moreover, alcohol induced liver damage or alcoholic liver disease has also become a prominent cause of liver cirrhosis. Down regulation of liver Galβ1,4GlcNAc α2,6-sialyltransferase and thus lowering of sialylated form of plasma proteins has recently been found to be associated with alcohol induced liver damage/alcoholic liver cirrhosis which are mostly considered as the prelude to HCC.[18,19] Therefore, for better management and surveillance of HCC, early assessment of alcohol abuse that leads to LC can serve as a crucial step forward.[32] Our plasmonic detection platform can be an excellent addition to the existing diagnostic framework for the rapid, cheap and easy routine assessment of alcohol abuse leading to LC.

Electronic Supplementary Information (ESI)

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.XXXX/an.2015.XX.XXX.

Acknowledgements

PPB thanks Department of Science and Technology (DST), India for Fast Track Young Scientist-DST-SERB project (SB/FTP/CS-017/2013) and Bose Institute, Kolkata for research infrastructure. BPC wishes to thank Indian Science Congress Association for 'Sir Asutosh Mookerjee fellowship' [2310/73/2013-2014]. We are thankful to Dr. Y. K. Chawla, Director, PGIMER, Chandigarh, India for his helpful advice. Authors declare no conflict of interest in this work**.**

Notes and References

1 C. I. Balog, K. Stavenhagen, W. L. Fung, C. A. Koeleman, L. A. McDonnell, A. Verhoeven, W. E. Mesker, R. A. Tollenaar, A. M. Deelder and M. Wuhrer, *Mol. Cell. Proteomics.,* 2012, **11,** 571–585.

2 A. Mehta, P. Norton, H. Liang, M. A. Comunale, M. Wang, L. Rodemich-Betesh, A. Koszycki, K. Noda, E. Miyoshi and T. Block, *Cancer. Epidemiol. Biomarkers. Prev.*, 2012, **21,** 925–933.

3 M. L. deLeoz, L. J. Young, H. J. An, S. R. Kronewitter, J. Kim, S. Miyamoto, A. D. Borowsky, H. K. Chew and C. B. Lebrilla, *Mol.Cell. Proteomics.,* 2011, **10,** M110.002717-1.

4 R. L. Renee, S. Miyamoto and C B Lebrilla, *Mol. Cell. Proteomics.* 2013, **12,** 846-855.

5 S. R. Kronewitter, M. L. De Leoz, J. S. Strum, H. J. An, L. M. Dimapasoc, A. Guerrero, S. Miyamoto, C. B. Lebrilla and G. S. Leiserowitz, *Proteomics*, 2012, **12,** 2523–2538.

6 D. J. Harvey, *Proteomics*, 2005, **5,** 1774–1786.

7 M. Guillard, J. Gloerich, H. J. Wessels, E. Morava, R. A. Wevers and D. J. Lefeber, *Carbohydr. Res.,* 2009, **344,** 1550–1557.

8 J. Bruix, L. Boix, M. Sala, and J. M. Llovet, *Cancer. Cell*. 2004, **5,** 215-219.

9 M. C. Kew, *J. Hepatocellular. Carcinoma*., 2014, **1,** 115–125.

10 Y. F. Liaw, *Semin. Liver. Dis*., 2005, **25,** 40-47.

11 K. Koike, *Clin. Gastroenterol. Hepatol.* 2005, **3,** S132-S135.

12 F. X. Bosch, J. Ribes, and J. Borras, *Semin. Liver. Dis*. 1999, **19,** 271-285.

13 E. Caturelli, F. Bartolucci, E. Biasini, M. L. Vigliotti, A. Andriulli, D. A. Siena, V. Attino and M. Bisceglia, *Am. J. Gastroenterol*. 2002, **97,** 397-405.

14 L. Bolondi, J. Hepatology., 2003, **39,** 1076-1084.

15 B. Daniele, A. Bencivenga, A. S. Megna and V. Tinessa, *Gastroenterol*, 2004, **127**, S108-S112.

16 R. Tateishi, S. Shiina, H. Yoshida, T. Teratani, S. Obi, N. Yamashiki, H. Yoshida, M. Akamatsu, T. Kawabe and M. Omata, *Hepatol.* 2006, **44,** 1518-1527.

17 A. Colli, M. Fraquelli, G. Casazza, S. Massironi, A. Colucci, D. Conte and P. Duca, *Am. J. Gastroenterol.* 101 (2006) 513-523.

18 J. B. Burgess, J. U. Baenzigearn, W. R. Brown, *Hepatol.* 1992, **15,** 702-706.

19 M. Gong, M. Garige, K. Hirsch and M. R. Lakshman, *Metabol. Clin. Exp.,* 2007, **56,** 1241–1247.

20 H. Shu, X. Kang, K. Guo, S. Li, M. Li, L. Sun, L. Gan, Y. Liu and X .Qin, *Oncol. Rep.* 2010, **24,** 1271–1276.

21 S. Zhang, H. Shu, K. Luo, X. Kang, Y. Zhang, H. Lu and Y. Liu, *Mol. Biosyst.*, 2011, **7,** 1621–1628.

22 H. Shu, S. Zhang, X. Kang, S. Li, X. Qin, C. Sun, H. Lu and Y. Liu, *Acta. Biochim. Biophys. Sin.* 2011, **43,** 528–534.

23 M. Nakano, T. Nakagawa, T. Ito, T. Kitada, T. Hijioka, A. Kasahara, M. Tajiri, Y. Wada, N. Taniguchi and E. Miyoshi, *Int. J. Cancer.* 2008, **122**, 2301–2309.

24 T. Fujimura, Y. Shinohara, B. Tissot, P. C. Pang, M. Kurogochi, S. Saito, Y. Arai, M. Sadilek, K. Murayama, A. Dell, S. Nishimura and S. Hakomori, *Int. J. Cancer.* 2008, 39–49.

25 R. de la Rica and M. M. Stevens, *Nat. Nanotech.* 2012, **7**, 821–824.

26 P. D. Howes, S. Rana, and M. M. Stevens, *Chem. Soc. Rev*. 2014, **13**, 3835-3853.

27 M. Liljeblad, A. Lundblad and P. Pahlsson, *Biosens. Bioelectron.* 2002, **17**, 883-891.

28 M. C. Bordasa, N. S. Serbource-Goguela, J M Fegera, J. M. Maccariob, J. M. Agneraya and G. M. Durand, Clin. Chim. Acta. 1982, **125,** 311-318.

29 S. C. Gordon, L.E. Lamerato, L.B. Rupp, S.D. Holmberg, A.C. Moorman, P.R. Spradling, E. Teshale, F. Xu, J. A. Boscarino, V. Vijayadeva, M.A. Schmidt, N. Oja-Tebbe and M.Lu, *Am. J. Gastroenterol.* 2015, doi: 10.1038/ajg.2015.203

30 D. C. Rockey, S. H. Caldwell, Z. D. Goodman, R. C. Nelson, and A. D. Smith, Liver biopsy. *Hepatology* 2009, **49**, 1017–1044.

31 G. L. Davis, M. J. Alter, H. El-Serag, T. Poynard, and L. W. Jennings, *Gastroenterology*, 2010, **138,** 513-521.

32 E. Gruszewska, B. Cylwik, A. Panasiuk, M. Szmitkowski, R. Flisiak, and L. Chrostek, *Biomed. Res. Intl.* 2014, 876096.

Figure legends

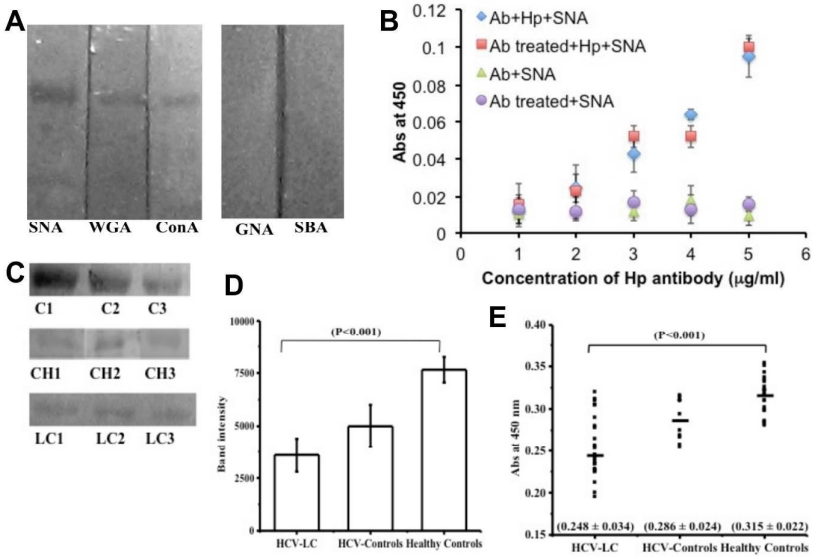


Fig. 1 A) Binding profile of commercial Hp with different lectins (SNA, WGA, ConA, GNA and SBA) as determined by lectin blot. B) Standardization of non-treated and treated mAb-Hp concentration used for the assay of serum Hp sialylation with SNA. C) Sialylation level of Hp in healthy controls, HCV-controls and HLC-LC patients groups using sialic acid specific lectin, SNA by lectin blot. In healthy controls (n=20), C1, C2 and C3 represent pool consisted of 3 samples each. Similarly, in HCV-controls group (n = 10) CH1, CH2 and CH3 represent pool consisted of 3 samples each and in HCV-LC patient group (n = 30) LC1, LC2 and LC3 represent pool consisted of 3 samples each. D) Mean Band intensity of serum Hp level of pooled samples from HCV-LC, HCV-controls and healthy controls as measured by Image J software. E) Difference in SNA reactivity of serum Hp with in healthy controls, HCV-controls and HCV-LC patient groups estimated by ELISA. The horizontal line indicates the mean value of Hp binding with SNA.

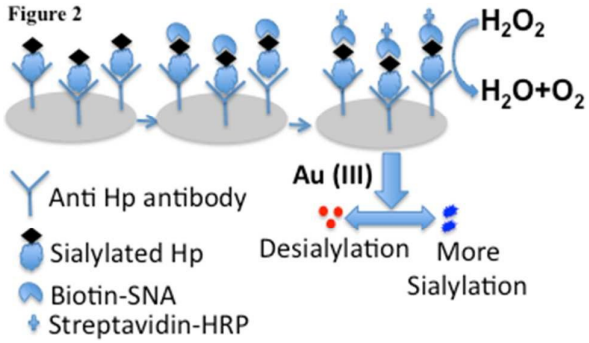


Fig. 2 Schematic representation of the detection of the extent of α 2,6-sialylation of serum Hp by plasmonic ELLSA.

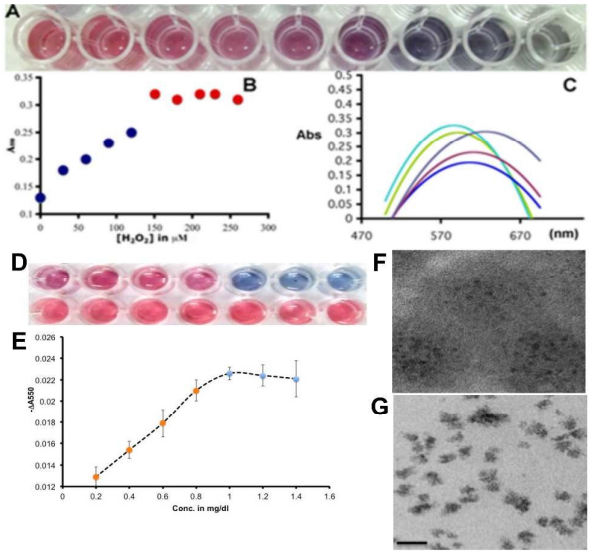
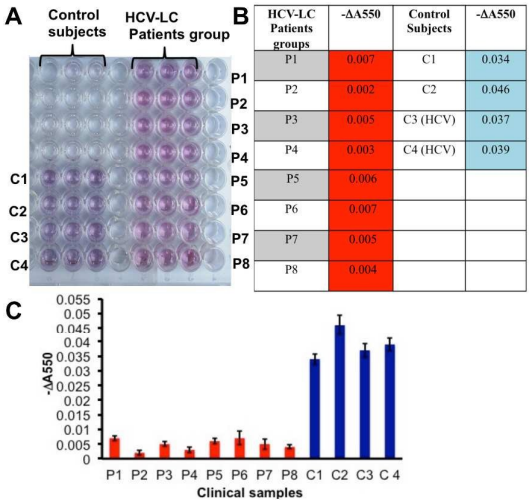


Fig. 3 A) With increasing concentration of reducing agent (H_2O_2) to 0.2 mM Au(III) the color changes from blue to red (30, 60, 90, 120, 150,180, 210, 240, 270 µM final concentration in wells from right to left). At 120 µM concentration of H_2O_2 theintermediate color of red and blue was observed showing the beginning of aggregation of GNP. B) Absorbance at 550 nm increases from blue to red particles and above 150 µM hydrogen peroxide red particles had very little differences ofabsorbances at 550 nm. C) Spectral shifts for different colored particles: 568 nm for red particles, ~645 nm for blue particles. D) Visual detection of Hp sialylation level by the generation of blue and red colored GNP with varying concentration of Hp in top lane compared with the same concentration of BSA in bottom lane. The increasing concentration of Hp/ BSA has been 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6 mg/dl from left to right. E) The calibration curve was showing the decrease in the difference in absorbance at 550 nm (-ΔA550 = -(A550 for Hp - A550 for BSA)) for Hp at different concentrations with respect to BSA at same concentration. The color change from red to blue was occurring in the range of ~ 1.0 mg/dl. F) Transmission electron microscopy (TEM) images of red, non-aggregated and blue, aggregated gold nanoparticles. Scale bar: 20 nm.



HCV-LC Patients groups	-ΔA550	Control Subjects	-ΔA550
P1	0.007	C1	0.034
P2	0.002	C2	0.046
P3	0.005	C3 (HCV)	0.037
P4	0.003	C4 (HCV)	0.039
P5	0.006		
P6	0.007		
P7	0.005		
P8	0.004		

Fig. 4 A) Determination of serum Hp sialylation in HCV-LC patient's group (P1, P2, P3, P4, P5, P6, P7 and P8), healthy controls (C1 and C2) and HCV-control (C3 and C4) subjects by plasmonic ELLSA. Experiment was performed in triplicate. B) The table showed the value of '-ΔA550', which correlated to the degree of Hp sialylation in serum samples. C) Graphical representation for comparative demonstration of the value of '-ΔA550' in the clinical samples (P for patients and C for controls).

Table 1: Diagnostic criteria of liver cirrhosis (HCV-LC) patients, healthy and viral hepatitis C (HCV) controls

Groups	Healthy Control (n=20)	HCV control (n=10)	HCV-LC (n=30)
Age (years)	25–65	22-58	34–69
Gender (M/F)	25/5	7/3	26/4
AFP (ng/ml)	26±2	49±19	94±55
ALT (IU/l)	5–40	37-75	63–138
AST (IU/l)	10–34	43-105	63–138
ALP (U/l)	25–110	74-341	98–526
Conjugated Bilirubin (mg/dl)	0.0-0.3	2.1-4.3	2.7–4.8
Albumin (g/dl)	3.5–5.5	2.7- 4.6	3.1–4.8
Prothrombin time (s)	11–13.5	15-19	16–21
Anti-HCV	-	+	+
Serum HCV RNA	-	(30-50) X10^5 Eq/ml	(15-60) X10^5 Eq/ml
MR and USG imaging of liver	Regular USG, showed normal size of liver and homogeneous parenchymal echo-pattern	Hepatomegaly with no significant changes in echogenicity, no-focal parenchymal lesion	Small, often multiple nodules of low signal intensity and of variable enhancement were characteristically observed.