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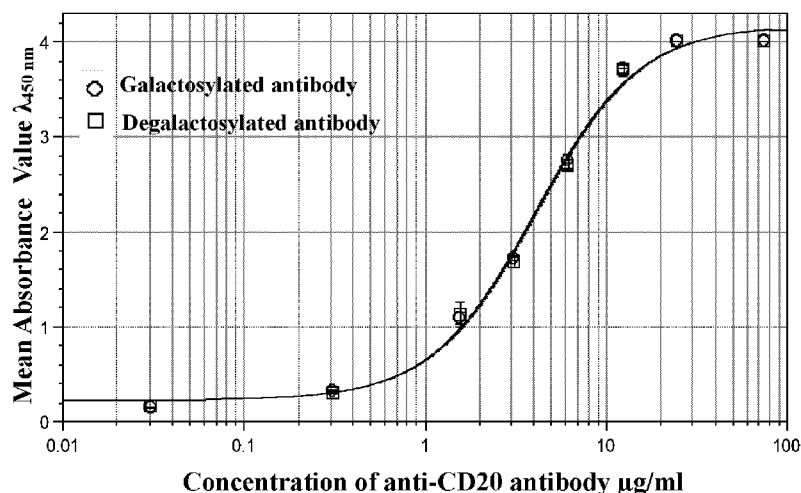
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(54) Title: IN-VITRO ASSAY FOR THE ASSESSMENT OF POST TRANSLATIONALLY MODIFIED PROTEIN ISOFORMS

**Figure 1**

(57) Abstract: The present invention discloses an in-vitro bioassay for distinguishing post translationally modified isoforms of a protein. In particular, the present invention discloses an in-vitro method for distinguishing post translationally modified isoforms of an antibody by C1q based ELISA involving a metal ion chelator.

IN-VITRO ASSAY FOR THE ASSESSMENT OF POST TRANSLATIONALLY MODIFIED PROTEIN ISOFORMS

RELATED APPLICATION

- 5 This application is related to and takes priority from Indian Provisional Application 179/CHE/2014 filed 15th January, 2014 and is herein incorporated in its entirety.

BACKGROUND OF INVENTION

- 10 Protein-protein interactions form the mainstay of vital biological processes like signal transduction, ligand receptor interaction, immune response etc. Often, such interactions are aided by post translational modifications (PTM) of proteins which involve attachment of functional groups, such as methyl, lipoyl, sulfate, phosphate and glycosyl to selected sites of a newly synthesized protein. These
15 groups impart physico-chemical properties essential for protein-protein interactions, macromolecular targeting, protein folding and solubility, and other vital cellular function.

- However, recombinant proteins, in particular immunoglobulins, are often expressed as different isoforms due to differential PTM. For instance,
20 immunoglobulins are glycosylated to varied extent in the Fc region giving rise to different glycoforms. Such PTM variations influence the binding of immunoglobulin to its downstream effectors - Fc receptor or a complement protein. Studies have demonstrated that galactosylated glycoforms facilitate better binding of the immunoglobulins to the complement protein, C1q. On the other
25 hand, glycovariants of immunoglobulins that lack galactose exhibit a diminished affinity to C1q. A diminished binding to C1q can lead to diminished complement mediated cytotoxicity exhibited by the immunoglobulin.

In other words, differences in PTM resulting in different glycoforms can differentially influence the outcome of a therapeutic protein preparation and may severely impact the therapeutic efficacy of the preparation.

Thus, assessment of such modifications by a functional ligand-receptor
5 assay is important. In a physiological system, low-affinity protein-protein interactions are characterized by fast binding kinetics and are rapidly reversible. These interactions often involve co-factors such as a 'metal ion' to aid the interaction and its subsequent binding affinity and kinetics. Roumenina et al. demonstrated that sequestering calcium by adding metal ion chelators like EDTA
10 diminishes the binding affinity between the immunoglobulin and C1q (Roumenina et. al., Biochemistry 2005, volume 44, pages 14097-14109).

Our studies revealed that standard ELISA based in-vitro ligand binding assays employed to assess metal ion aided protein-protein interactions, does not distinguish PTM isoforms in the protein, since the metal ion stabilized target
15 protein is bound indiscriminately by the different PTM isoforms of the interacting protein. In the present invention, significant differences between the relative binding affinities of the PTM isoforms to the metal-ion-stabilized target protein i.e., C1q, is obtained by suppressing the role of metal ion using a metal ion chelator. In other words, in the absence of the metal ion, the binding affinity of the
20 glycosylated isoforms of the protein to its target protein was seen to be relatively higher than that of the less or non-glycosylated isoforms.

Thus, the objective of the invention is to distinguish the PTM isoforms by monitoring the substantial difference in the relative binding affinities of the PTM isoforms to its target protein, wherein such difference is achieved by addition of a
25 metal ion chelator to metal ion mediated protein-protein interactions. The proposed invention is a simple and an inexpensive assay methodology to distinguish and quantify different PTM isoforms present in a mixture of protein.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1: Depicts the C1q binding assay performed without EDTA

Figure 2: Depicts the C1q binding assay in the presence of 20 mM EDTA on anti-CD20 antibody sample 1

5 Figure 3: Depicts the C1q binding assay in the presence of 20 mM EDTA on anti-CD20 antibody sample 2

SUMMARY OF THE INVENTION

10 The present invention discloses a method to distinguish post-translationally modified isoforms in a protein by a ligand-binding assay. The method involves the addition of a metal ion chelator to a protein-protein interaction whose interaction is facilitated by a metal ion. The said addition of the metal ion chelator suppressed the effect of the metal ion such that differential post-translational modifications in a protein could be distinguished and quantified.

15 In particular, the method distinguishes and quantifies differentially post-translated isoforms of a C1q binding protein or an antibody.

The method, as described above in the present invention is a functional assay that directly indicates the activity of a protein or an antibody.

20 DESCRIPTION OF THE INVENTION

Various embodiments of the disclosed invention provide a method of a ligand-binding assay to distinguish PTM isoforms of a protein, wherein the method involves the addition of a metal ion chelator. The assay is sensitive in distinguishing and quantifying different PTM isoforms of a protein, in particular
25 of an antibody, and in addition serves as a functional assay assessing the impact of

differential isoforms on the complement dependent cytotoxic activity of the antibody.

In an embodiment, the claimed invention discloses a ligand-binding assay to distinguish different PTM isoforms of a protein, wherein a metal ion chelator is added to the assay and wherein the said addition of the chelator results in a difference in the relative binding affinities of the PTM isoforms to its target protein.

In another embodiment, the target protein is a complement protein, C1q.

In an embodiment, the claimed invention discloses a ligand-binding assay to distinguish different PTM isoforms of a C1q binding protein, wherein a metal ion chelator is added to the assay and wherein the said addition of the chelator results in a difference in the relative binding affinities of the PTM isoforms to the C1q protein.

In an embodiment, the invention discloses a method for distinguishing differentially galactosylated antibody isoforms in a sample, comprising steps of;

- a) adding a buffer comprising C1q and a metal ion chelator to the sample
- b) incubating the said sample with the buffer of step a) and,
- c) assaying the binding of C1q to the antibody in the sample,

wherein, the differential galactosylations of antibodies in the sample is reflected in the differential binding of C1q to the antibodies.

In an embodiment, the invention discloses a method for distinguishing differentially galactosylated antibody isoforms in an antibody mixture, comprising steps of;

- a) adding a buffer comprising C1q and a metal ion chelator to the antibody mixture
- b) incubating the said mixture with the buffer of step a) and,
- c) assaying the binding of C1q to the antibody mixture,

wherein, the differential galactosylations of antibodies in the mixture is reflected in the differential binding of C1q to the antibodies.

In another embodiment, the antibody is an anti-CD20 antibody.

In another embodiment, the metal ion chelator is ethylene diamine tetra
5 acetic acid (EDTA).

The said inventive method of addition of a metal ion chelator to a ligand-binding assay, to suppress the effect of a metal ion and decrease the original affinity of the protein protein interaction, resulting in distinguishing different PTM isoforms of a protein, may be performed in any low affinity protein-protein
10 interaction ($K_d > 1 \mu\text{M}$) whose interaction is facilitated by a metal ion.

A metal ion chelator mentioned in the embodiments refers to a compound which sequesters or complexes a metal ion or reduces its effect in a system. Commercially available chelators include, but are not limited to compounds such as EDTA, Ethylene glycol tetra acetic acid (EGTA), Humic acid or Fulvic acid, or
15 functional derivatives thereof.

The term “post translationally modified (PTM) isoforms” as used herein refers to a protein composition having varied isoforms as a result of differential post translational modifications.

C1q is a complement protein and the term “C1q binding protein” refers to
20 a protein interacting with C1q.

The invention is more fully understood by reference to the following examples. These examples should not, however, be construed as limiting the scope of the invention.

EXAMPLE 1

25 An anti-CD20 antibody was cloned and expressed in a CHO cell line as described in U.S. Patent No. 7,381,560, which is incorporated herein by reference. The cell culture broth containing the expressed antibody was purified using

combination of affinity and ion exchange chromatography and purified samples containing anti-CD20 antibody and/or isoforms of anti-CD20 antibody were obtained.

Two different samples of recombinant anti-CD20 antibody were taken and
5 a complement, C1q, based ELISA was performed on galactosylated and completely degalactosylated isoforms. Degalactosylated immunoglobulins were obtained by treating them with degalactosidase enzyme (QA-Bio) and incubating at 37 °C for 4 h.

Immunoglobulins in 1X phosphate buffered saline (PBS), pH 7.4, were
10 coated to the 96 wells ELISA plate in varying amount at 37 °C with shaking at 300 rpm. Subsequently, the plate was washed with PBS containing 0.05 % tween (Sigma Aldrich) (PBST) and the nonspecific sites were blocked using skimmed milk powder in 1XPBS at RT for 2 h. This was again followed by a washing step with 0.05 % PBST. Human C1q protein (Sigma life sciences), diluted in 1XPBS,
15 was added to each of the wells and incubated at 25 °C for 1 h. with shaking at 300 rpm. The wells were again washed with 0.05 % PBST and the detection was done by adding Sheep anti-C1q polyclonal antibody (Abcam) tagged with horseradish peroxidase HRP and incubating at 25 °C for 1 h with shaking at 300rpm followed by a washing step. The substrate, 3, 3', 5, 5'-Tetramethylbenzidine (TMB)
20 (Surmodics), was added to the wells and the plate was incubated at RT for 15 minutes in dark. The reaction was stopped by adding TMB stop solution (Surmodics) to each well and the absorbance of the color developed was recorded using ELISA plate reader (Molecular Devices) at 450 nm. The mean absorbance of each well was plotted against the respective antibody concentration on a semi
25 log scale.

EXAMPLE 2

For the detection of differentially galactosylated immunoglobulins, the assay was performed using the procedure as described in Example 1 except that,

C1q was diluted in a buffer containing 20 mM EDTA. Percent relative potency of the galactosylated and degalactosylated samples were computed and compared.

CLAIMS:

We Claim:

1. A ligand binding assay for differential binding interactions of a ligand with a target protein, wherein the said ligand and the target protein differentially
5 interacts in the presence of a chelator.
2. The ligand binding assay as per claim 1, wherein the target protein is an Fc containing protein and the ligand is an Fc binding protein.
3. The ligand binding assay as per claim 1, wherein the ligand is complement protein C1Q.
- 10 4. The ligand binding assay as per claim 1, wherein the chelator is a metal ion chelator.
5. A ligand binding assay for differential binding interactions of a ligand with a target protein, wherein the ligand binds differentially to different isoforms of a target protein in presence of a chelator.
- 15 6. The method as per claim 5, wherein the isoforms of the target proteins are post translational modifications.
7. The method as per claim 5, wherein the isoforms of the target proteins are glycosylated isoforms.
8. The method as per claim 5, wherein the chelator is a metal ion chelator.
- 20 9. A ligand binding assay for distinguishing differentially galactosylated antibody isoforms in a sample comprising steps of
 - a. adding a buffer comprising C1q and a metal ion chelator to the sample
 - b. incubating the said sample with the buffer of step a) and
 - 25 c. assaying the binding of C1q to the antibody in the sample.

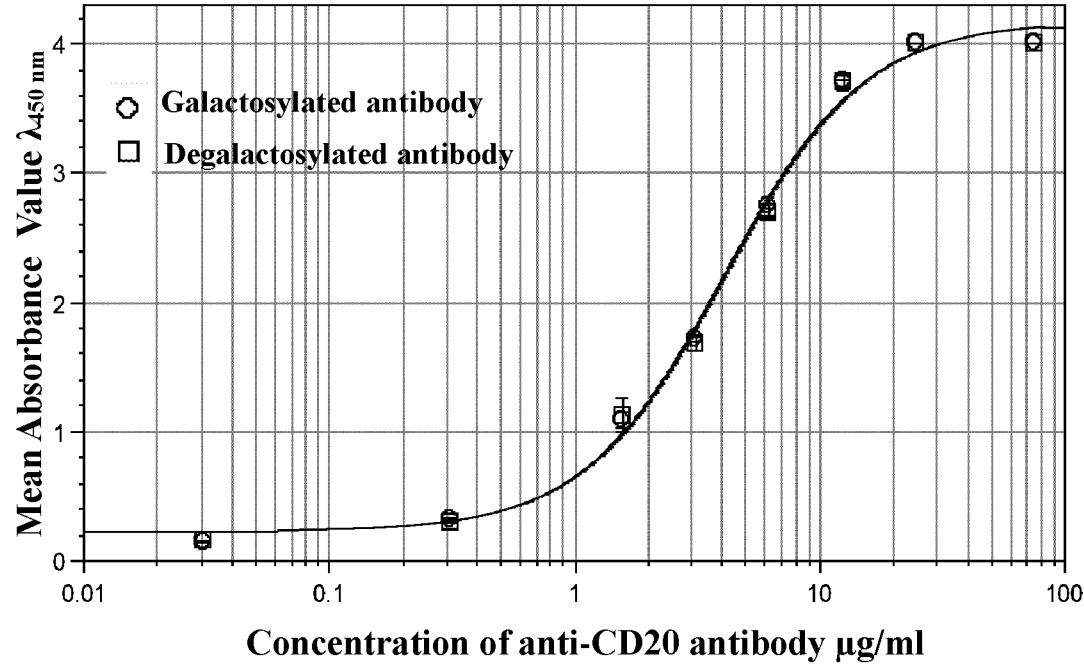


Figure 1

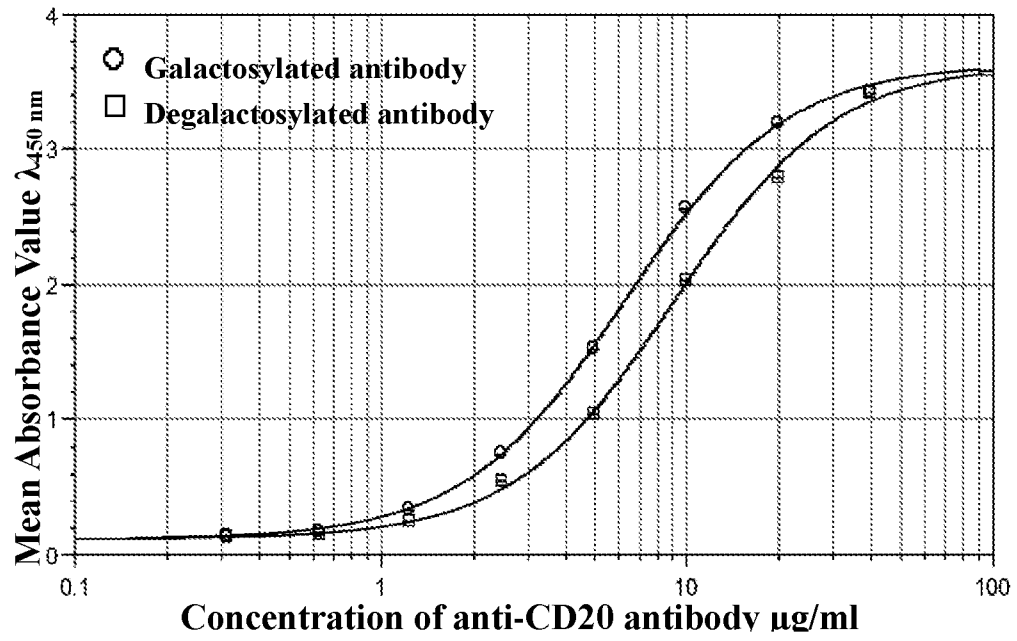
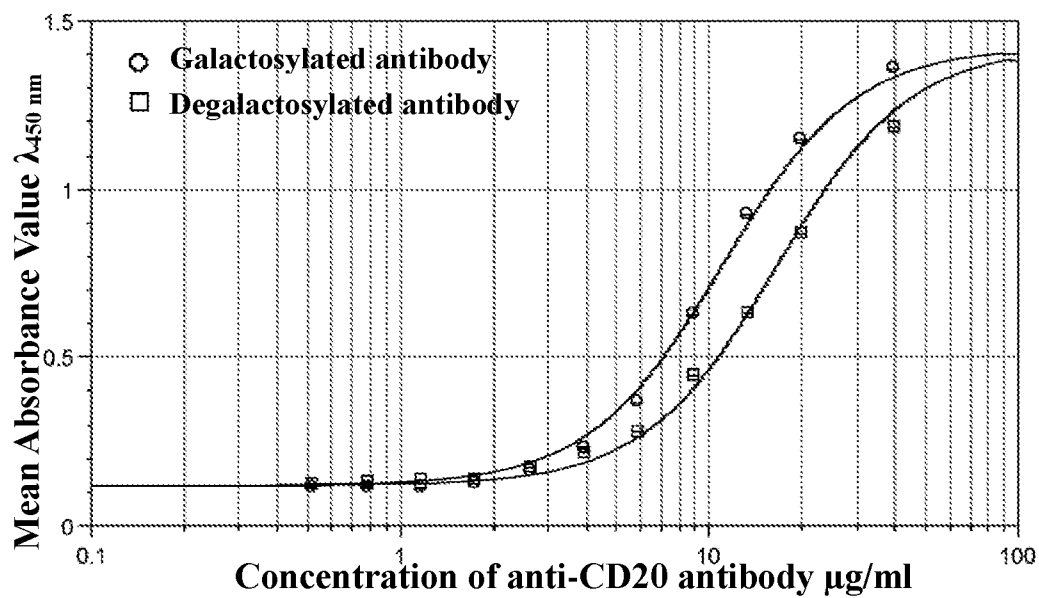


Figure 2

**Figure 3**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/IB2015/050212

A. CLASSIFICATION OF SUBJECT MATTER

G01N 33/53(2006.01)i; G01N 33/541(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CNABS,CNKI,NCBI,VEN,ISI-WEB OF KNOWLEDGE:metal ion, chelator, chelate, chelating, antibody, post translational, modified, PTM, distinguish, isoform, binding, affinity, EDTA, C1q, galactosylated, target, ligand, interaction

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Martin Ďerný et al. "Advances in purification and separation of posttranslationally modified proteins" <i>JOURNAL OF PROTEOMICS</i> , Vol. vol.92, 15 June 2013 (2013-06-15), ISSN: pages 2-27, See the abstract, and page 17	1-9
A	Yan Wang et al. "A Strategy for Direct Identification of Protein S-Nitrosylation Sites by QuadrupoleTime-of-Flight Mass Spectrometry" <i>J Am Soc Mass Spectrom</i> , Vol. vol.19, No. issue 9, 20 June 2008 (2008-06-20), ISSN: pages 1353-1360, See pages 1355 and 1356	1-9

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

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