Heterogeneous and Homogeneous Time-resolved Fluorescence-Based Assays for a Low-Affinity Binding Reaction

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Introduction

Low-affinity binding reactions are often problematic due to either a poor signal-to-background ratio or a need to use high concentrations of labeled components. We set up heterogeneous (DELFIA®) and homogeneous (LANCE®) assay formats for carbohydrate-lectin interaction to determine the applicability of time-resolved fluorometry in binding reactions with close to micromolar affinity. We used both a biotinylated univalent and a biotinylated multivalent D-mannose as a carbohydrate component. In DELFIA assays biotinylated D-mannose derivatives were immobilized to streptavidin coated plate and incubated with Eu-labeled concanavalin A (ConA). Different approaches for a LANCE assay were tested and here we present the approach of using Eu-labeled streptavidin, Alexa647-labeled ConA and biotinylated D-mannose.
Methods

Assays were performed using both a univalent and a multivalent biotinylated α-D-mannose (GlykoTech) as target carbohydrates. Multivalent biotinylated α-D-mannose is a polyacrylamide polymer with an average molecular weight of approximately 30 000 and contains 10 biotin moieties and 40 mannose residues in each molecule on the average.

DELFIA assay
Displacement assays using α-D-glucose and D-mannose as competitors were performed in clear streptavidin coated 96-well microtitration plates. Univalent and multivalent biotinylated D-mannose derivatives were diluted to concentrations of 30 nM and 2 nM, respectively, in DELFIA Assay buffer and then incubated in streptavidin coated wells (200 µL/well). After one hour incubation wells were washed once. Then different concentrations of D-glucose and D-mannose (in triplicates) were incubated in the wells together with 5 nM Eu-N1-ConA in lectin binding buffer (50 mM Tris-HCl, pH 7.5, containing 50 mM NaCl, 2 mM CaCl₂, 0.5% BSA, 0.05% Tween 20, and 50 µM EDTA). After a 4-hour incubation at RT wells were washed six times using DELFIA Wash solution. Next DELFIA Enhancement solution (200 µL/well) was added and plate was shaken for 5 minutes followed by the measurement in VICTOR Multilabel counter using the factory-set Eu protocol.

LANCE assay
Competition assays were performed in clear 96-well plates and white 384-well plates. Assay format (Figure 1) employed different concentrations of D-glucose and D-mannose (in duplicates), 6 nM biotinylated multivalent D-mannose, 6 nM Eu-W1024-streptavidin and 40 nM Alexa-647-labeled ConA in the lectin binding buffer (same as above without EDTA). After incubating at RT for one hour plates were measured in VICTOR Multilabel counter using the factory-set LANCE protocol.

Figure 1. Principle of LANCE assay using the multivalent D-mannose derivative.
Results

Figure 2. DELFIA displacement curves using the univalent biotinylated D-mannose.

Figure 3. DELFIA displacement curves with the multivalent biotinylated D-mannose.
DELFIA assay worked well with both the univalent and the multivalent biotinylated D-mannose derivatives giving sigmoidal competition curves. Binding of Eu-N1-ConA to the univalent biotinylated D-mannose was inhibited with D-glucose and D-mannose giving IC50 values of 5.7 and 1.1 mM, respectively. DELFIA assay with the multivalent biotinylated D-mannose produced IC50 values of 3.5 and 0.6 mM for D-glucose and D-mannose, respectively. These assays gave signal-to-background ratios of 40 with the univalent and 120 with the multivalent biotinylated D-mannose. Increasing the amount of immobilized multivalent D-mannose resulted in S/B between 500 and 1000 (data not shown).

Homogeneous LANCE assay using the multivalent biotinylated D-mannose was also competitively inhibited with D-glucose and D-mannose giving IC50 values of 2.6 and 0.5 mM, respectively. This assay gave a S/B of about 10 while an assay with 10 nM Eu-W1024-streptavidin and biotinylated multivalent D-mannose together with 50 nM Alexa647-ConA gave a S/B of 25 (data not shown). The univalent biotinylated D-mannose also produced specific signal in the LANCE assay (data not shown). However, specific signal wasn’t stable and decreased already after 15 min incubation and after 2 hours there was no specific signal left. Increasing reagent concentrations up to 200 nM didn’t improve signal stability. We are optimizing assay conditions in order to get a stable signal also with the univalent biotinylated D-mannose.

Figure 4. LANCE competition curves using the multivalent biotinylated D-mannose.
Conclusions

Our results show that time-resolved fluorescence-based assays can be used in low-affinity binding reactions. The obtained IC50 values are in good agreement with the published values. Heterogeneous DELFIA assays worked well with both univalent and multivalent biotinylated D-mannose. Homogeneous LANCE assay gave reliable results with the multivalent carbohydrate target. The univalent biotinylated D-mannose produced a specific signal in the LANCE assay but this signal wasn’t stable in the described conditions.