

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 (DELFI[®]) and homogeneous (LANC[®]) 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.

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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.

DELFI^a 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 μ L/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 μ M EDTA). After a 4-hour incubation at RT wells were washed six times using DELFIA Wash solution. Next DELFIA Enhancement solution (200 μ L/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.

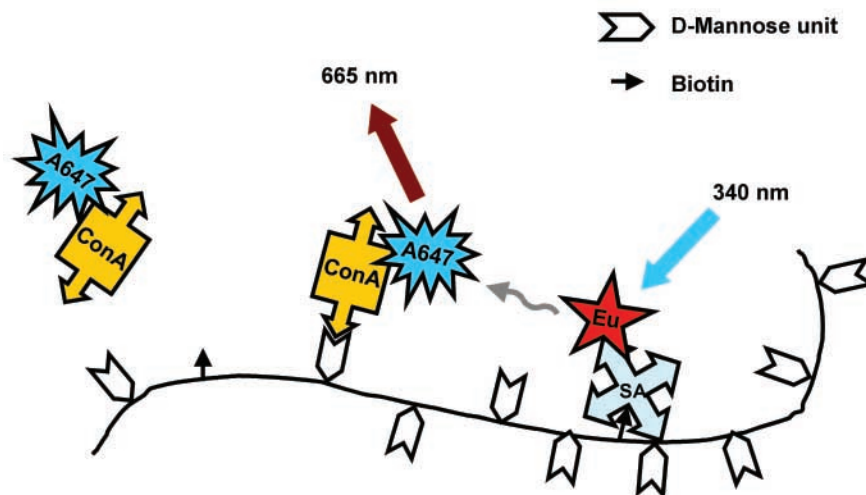


Figure 1. Principle of LANCE assay using the multivalent D-mannose derivative.

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Results

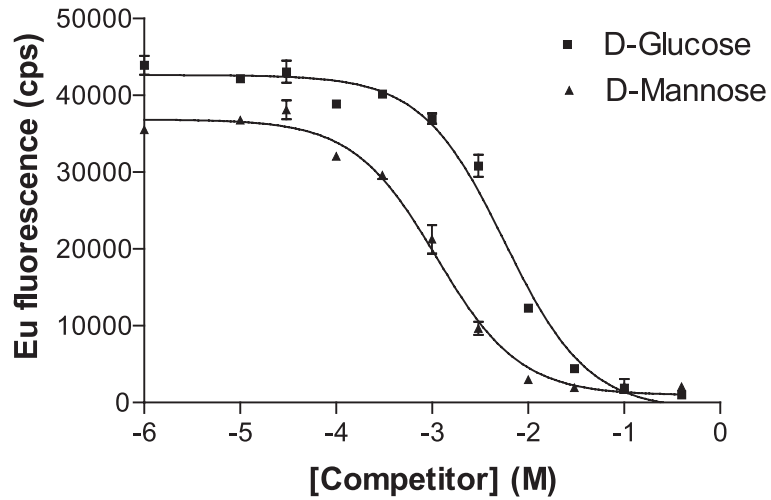


Figure 2. DELFIA displacement curves using the univalent biotinylated D-mannose.

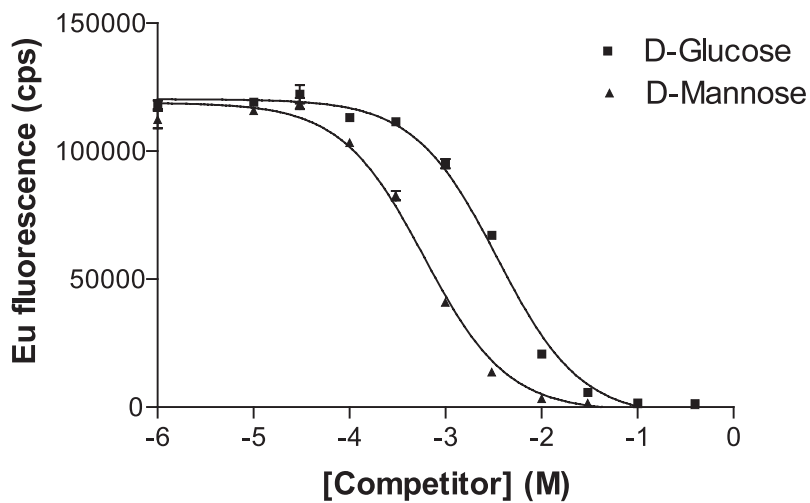


Figure 3. DELFIA displacement curves with the multivalent biotinylated D-mannose.

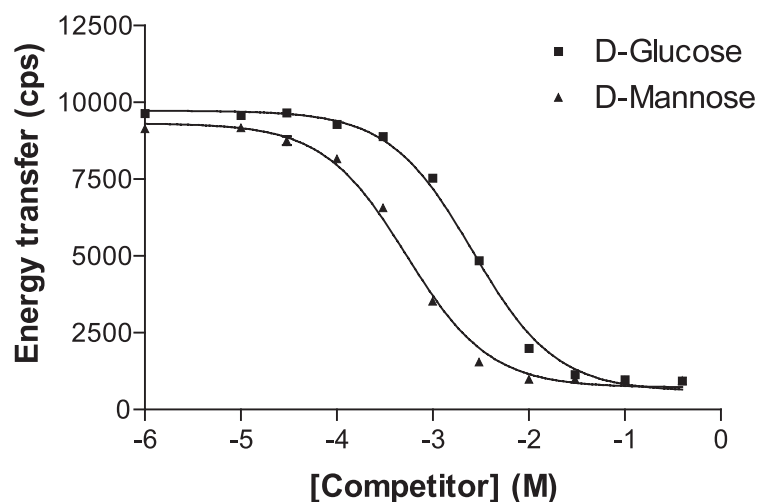


Figure 4. LANCE competition curves using the multivalent biotinylated D-mannose.

DELFLIA 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. DELFLIA 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.

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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.



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