

Development of a No Wash Cell Adhesion Assay for Screening

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Abstract

Cell-cell and cell-matrix binding assays have been difficult to develop and deploy in screening formats. Cell adhesion assay methods require multiple steps, labeling of the cells and repeat washing of the cells to determine specifically bound cell numbers. All of these steps contribute to poor reproducibility. A label-free, no wash assay has been developed using the BIND[®] Reader and 384-well BIND[®] Biosensors to screen and characterize the specific interaction between the vascular cell adhesion molecule-1 (VCAM-1) and the α 4 β 1 integrin, VLA-4, natively expressed on J6 Jurkat cells. Assay optimization including cation dependency and characterization of antagonists will be presented.

Introduction

Cell-cell and cell-matrix interactions are critical biological interactions involved in the development and the maintenance of tissues and organs. Cell adhesion molecules are cell surface membrane proteins that mediate and control these adhesive processes. These molecules not only function by linking cells to each other or to components of the extracellular matrix, but they also function as receptors that interact via their cytoplasmic domain with numerous signaling molecules including protein kinases and phosphatases (1, 2), G-proteins (3), or proteins of the β -catenin/armadillo family (4). During the last decade it has been recognized that defects of cell adhesion molecules and adhesion-linked signaling molecules are the molecular basis of various types of disease including cancer, infection and inflammation. This has resulted in an increased interest in cell adhesion assays that can be applied to both high throughput screening and compound profiling

BIND[®] Technology

BIND[®] from SRU Biosystems is a universal assay system that enables label-free detection of drug-target interactions. The system is comprised of the BIND[®] Reader and 96-, 384- or 1536-well microplate BIND[®] Biosensors. The BIND system takes advantage of a novel optical effect to provide very sensitive measurements of changes in binding or adherence in the proximity of the biosensor surface. The biosensor

incorporates a proprietary nanostructured optical grating, and is incorporated in microwell plates in industry standard formats (5). The grating of the BIND Biosensor reflects only a single wavelength ("Peak Wavelength Value" or "PWV"). When a cell attaches to the biosensor surface, this reflected wavelength increases (Figure 1). Real time attachment can be observed by measurement of the shift in PWV over time. For example, when cells are seeded onto an appropriately coated biosensor surface, cell attachment can be detected without the use of a label. Each addition to an assay, such as an agonist or antagonist, can be observed in real time to monitor the effect on cell adhesion to the surface.

Figure 1

Materials and Methods

All assay measurements were carried out using the BIND Reader, 96- (TiO-96-M) and 384-well (TiO-384-M) TiO BIND® Biosensors (SRU Biosystems). Multidrop 96 & 384 (Thermo Fisher Scientific) was used for plate washing, 96 & 384 tip CyBi Well (CyBio) was used for liquid additions.

J6 Jurkat Cells (ATCC) are grown and harvested according to the vendor recommended protocols. Vendor recommended media was used for growth of the cells and the assay media was Hanks balanced saline with 50 mM HEPES. Blocking buffer was 3% BSA (Sigma A-3912) in PBS. Wash buffer during surface preparation was PBS.

VCAM-ZZ was produced by GSK. 20 µl of IgG (Sigma, I-4506) in PBS at 50 µg/ml was added to the 96- or 384-well TiO BIND Biosensor plate and incubated for 3 hours at 37°C followed by washing with PBS. VCAM-ZZ (20 µl at 4 µg/ml) was added in the presence of 3% BSA and incubated overnight at 4°C followed by washing with PBS. All buffers were filtered before use.

Compounds were supplied in DMSO by GSK. The compounds were diluted in assay media to give a final DMSO concentration of less than 0.2% for dose response determination.

Assay Development

Label-free microplate-based assay systems have an advantage over standard assay methods when carrying out assay development. Where standard assay development methods only provide an end-point measurement once all steps of a multi-step assay have been carried out, label-free assay development allows one to monitor all stages of the assay in real time. By following the plate coating steps, the label-free method enables the optimization of reagent use and incubation time. Having a quantitative measure of the plate coating steps also enables better quality control within and between assay runs.

For this assay, development was first carried out in the 96-well TiO BIND Biosensor and then the assay was easily miniaturized to 384-well format using one third the number of cells. Testing of multiple assay parameters were carried out in a 96-well BIND Biosensor plate. The parameters tested were immobilization of VCAM-ZZ (coating pH, direct absorption, orientation via IgG capture), plate blocking, order of reagent addition, cell number and centrifugation of plate.

Figure 2 demonstrates that addition of EDTA gives >90% reduction in cation-dependent cell adhesion signal. Moreover, EDTA reversed binding that had already occurred (Fig 3). With a BSA blocked surface, VCAM-specific binding was 75% of the total signal. The order of addition of cells and

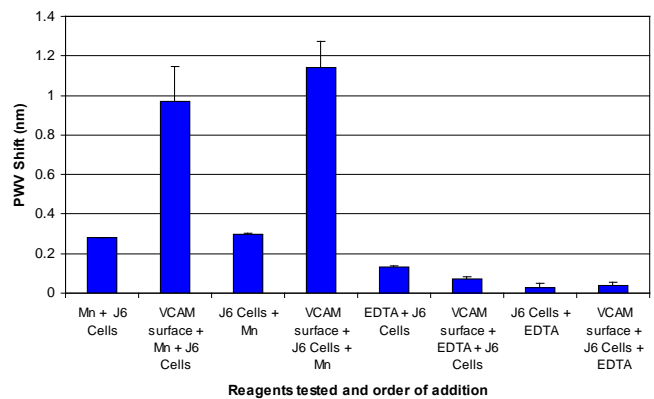


Figure 2: Order of addition of cells and cation has no effect on VCAM dependent binding. VCAM was captured via IgG coated on the 96-well TiO BIND Biosensor, blocked with 3% BSA, washed and then either 150,000 J6 cells were added followed by Mn^{++} (1mM) and/or EDTA (4 mM). The order of addition of Mn^{++} followed by cells was also tested. Direct coating of VCAM on the biosensor was also tested but orientation via IgG surface coating gave superior results (data not shown).

cation did not affect the maximum signal obtained but the time-courses revealed (data not shown) that with addition of cells after Mn^{++} addition a lag in binding occurred. Once the coated and blocked biosensor was prepared, the remainder of the assay was carried out without the use of wash steps, thus improving precision and scalability to a screening mode. This simplified the workflow and improved reproducibility over the existing cell adhesion assay.

Best assay conditions

- No wash required after activating the adhesion
- Capture of VCAM-ZZ via IgG capture improved assay signal
- Immobilization of VCAM-ZZ at pH 7.4, 3hrs at 37C
- 3% BSA block reduced VCAM-independent binding of cells
- Addition of cation after cells have settled, simplified assay workflow and shortened the time to maximum binding

Titration of Cation and Reversibility of Binding

The optimization of cation concentration was carried out in 384-well TiO BIND Biosensors (Figure 3). In the range up to 0.25mM Mn^{++} , saturable binding of J6 cells was seen with a max effect observed at a Mn^{++} concentration of <0.1 mM (EC50- 0.01mM, data not shown). Manganese concentrations above 0.25mM inhibited the cell binding response. Notably, using BIND, Mg^{++} also demonstrated saturable dose response that reached a similar maximum response to Mn^{++} . The EC50 or apparent Kd of Magnesium was determined to be 1.2 +/- 0.06 mM. This was consistent with physiological conditions for cell adhesion. With the legacy cell adhesion assay, it had not been possible to obtain robust magnesium dependent binding.

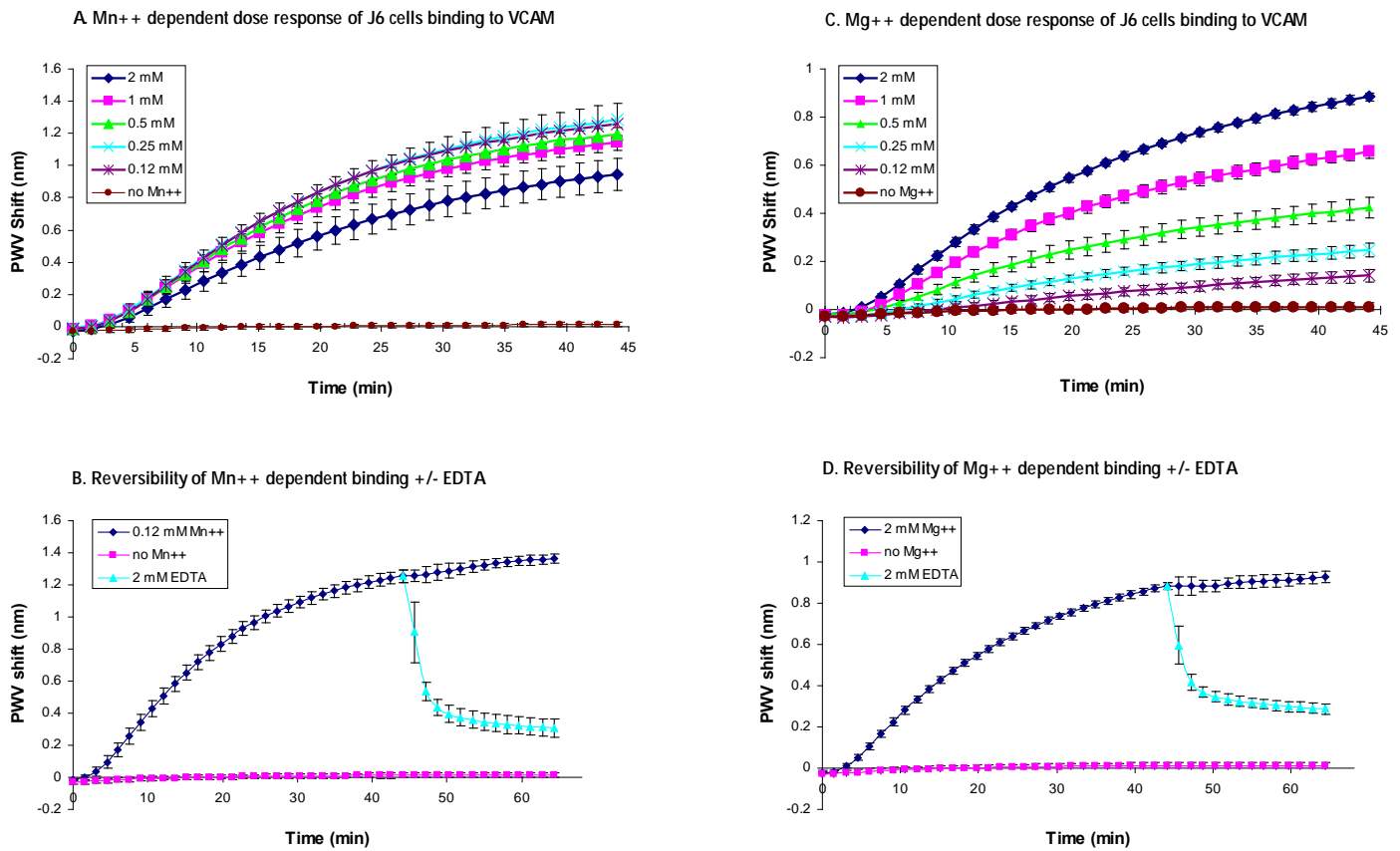


Figure 3: Cation dependent binding of 50,000 J6 cells to VCAM immobilized on the 384-well TiO BIND Biosensor. A. Mn^{++} dose dependent binding, B. Reversibility of Mn^{++} dependent binding in the presence of 2mM EDTA. C. Mg^{++} dose dependent binding, D. Reversibility of Mg^{++} dependent binding in the presence of 2mM EDTA.

Optimization of Cell Number

The maximum binding response increased with increasing cell numbers (see Figure 4). Often in cell adhesion assays better results are obtained when the assay plate is spun in a centrifuge after cell addition to ensure the cells settle quickly and evenly to the bottom of the plate, thus reducing some variability. It was determined on BIND that centrifugation did not improve the assay performance (data not shown) and had a negative effect in that there was an increase in the level of cation-independent binding.

IC50 determination of VCAM-VLA4 antagonists

A panel of small molecule antagonists of the interaction of VCAM with its ligand VLA-4 were tested in both the Mn^{++} and Mg^{++} dependent BIND assay formats. These compounds had been identified in a Mn^{++} dependent Opera assay because even at infinite Mg^{++} concentration there was insufficient signal for the legacy cell adhesion assay used. The IC50s obtained using BIND in replicate assays were very reproducible (see Table 1). BIND had a significant advantage over the legacy cell adhesion assay method in allowing the use

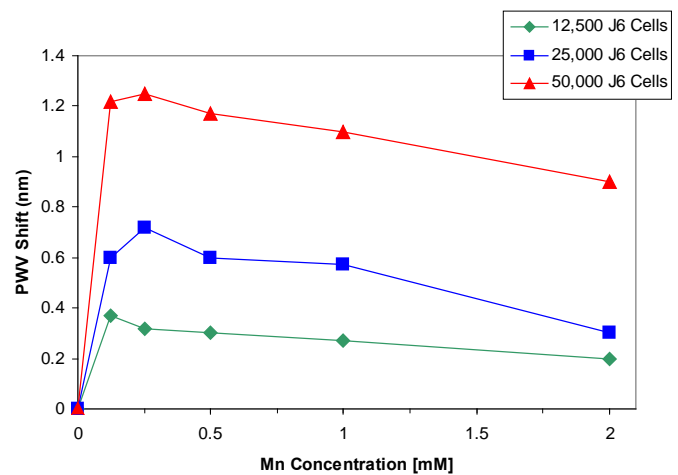


Figure 4: Titration of J6 cell number and Mn^{++} concentration. At each cell number/well tested the maximum binding was reached at the same Mn^{++} concentration of 0.1 mM

of physiological Mg^{++} , rather than the artificial use of Mn^{++} . The antagonists identified in the legacy assay proved to be more potent in the Mg^{++} dependent BIND assay versus the

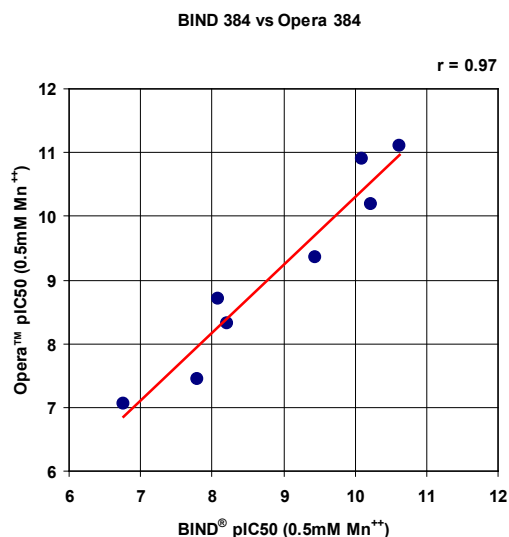


Figure 5: *A4b1* Integrin Antagonist pIC_{50} Value Correlation

Mn^{++} dependent BIND assay. This was an interesting novel result. The IC_{50} s obtained with Mg^{++} were too potent to determine with the utilized concentration range of antagonists. To fully quantify the differences in potency in Mg^{++} and in Mn^{++} , further experiments would be required in which full-dose-response curves for each antagonist were obtained in the presence of both sub-saturating and saturating Mn^{++} and Mg^{++} . The antagonist IC_{50} change between the two cations could be due to differences in how the ions activate the receptors and the clustering of the receptors on the cell surface. It is possible that the Mn^{++} causes a higher avidity for the VCAM/VLA-4 interaction, hence requiring higher concentrations of antagonist to abrogate binding. The rank order of potencies was similar to the legacy data (see Table 1), and there was an excellent correlation in pIC_{50} s over three orders of magnitude of potency (Figure 5). The BIND assay provides a robust signal, reproducible IC_{50} s over a large dynamic range and compounds were assessed to have greater potency than previously anticipated in physiological Mg^{++} .

Conclusions

- This assay, unlike the legacy assay, was easily miniaturized from 96- to a 384-well format to reduce cell numbers and protein requirements as well as to increase the throughput
- Excellent control of assay development, enabling all assay parameters to be investigated and optimized
- Homogenous assay conditions could be developed for not only a Mn^{++} dependent assay but also for Mg^{++} dependent cell-binding thus providing a more physiologically relevant assay with enhanced reproducibility

Compound ID	pIC ₅₀ (BIND) @ 0.5 mM Mn ⁺⁺		pIC ₅₀ (BIND) @ 4.0 mM Mg ⁺⁺		pIC ₅₀ (Opera) @ 0.5 mM Mn ⁺⁺
	Plate 1	Plate 2	Plate 1	Plate 2	
Antagonist F	>10.62	10.23	>10.62	>10.62	10.18
Antagonist E	9.67	9.24	>10.62	10.59	9.36
Antagonist C	>10.62	>10.62	>10.62	9.33	11.1
Antagonist B	>10.62	10.09	>10.62	>10.62	10.9
Antagonist A	7.64	7.94	>10.62	>10.62	7.44
Antagonist G	6.87	6.67	>10.62	9.26	7.05
Antagonist D	8.54	7.9	>10.62	>10.62	8.32
Antagonist H	8.25	7.91	>10.62	>10.62	8.71

Table 1: IC_{50} Determination of VCAM-VLA4 antagonists on J6 Cells (50K/well, 384)

- The high signal to background and the lack of any wash procedure allowed the BIND assay to quantify a larger dynamic range of avidity of cell adhesion than the legacy assay
- Antagonists could be identified in the Mn^{++} dependent BIND assay with highly reproducible IC_{50} s and an excellent correlation to a legacy assay. Additionally the Mg^{++} dependent BIND assay demonstrated greater antagonist potency

References

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