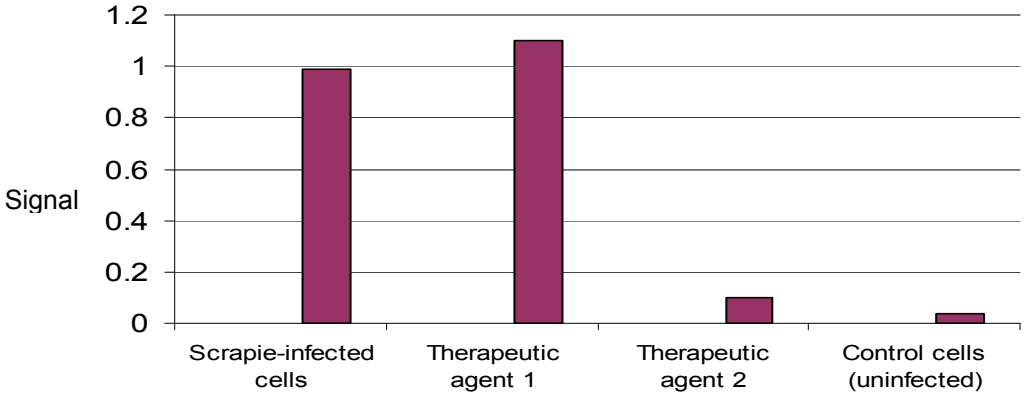
Example 4. Investigation of scrapie infected cell culture and treatment with potential therapeutic agents

Method

Neuronal cell cultures infected with scrapie were counted, lysed and the abnormal prion protein separated by **PAD-Beads** prior to assay by an in-house prion ELISA. The same neuronal cells which were uninfected were used as controls. In addition, infected neuronal cells that had been treated with therapeutic agents in the growth media were tested.

Results

In each assay 10^5 cells were tested. The abnormal prion protein could be clearly separated and detected by the ELISA in the scrapie infected cells. The signal in the cells treated with therapeutic agent 2 that was known to prevent prion protein aggregation in vitro clearly showed a reduction in abnormal prion protein which suggested that this agent did have a valuable therapeutic potential. Therapeutic agent 1 had little effect.



Conclusion

PAD-Beads can be used with cell culture systems to identify and characterise the effect of potential therapeutic agents.

microsens biotechnologies

Seprion™-PAD

Microsens Seprion Technology™

Tools for Protein Aggregation Disease Diagnostics

PAD-Beads

Magnetic Bead based Sample Preparation System for the Study of Protein Aggregation Diseases

Technical Manual and Protocols

(100 separations)

For research use only

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I. Introduction

PAD-Beads are a unique tool that can be used to separate abnormal proteins present in many types of Protein Aggregation Diseases from the normal unaggregated protein (Lane *et al.* 2003. Clinical Chemistry 49:1774-1775). This ability to easily and efficiently separate the abnormal from normal protein facilitates research on Protein Aggregation Diseases. Research applications include the study and monitoring of Protein Aggregation Diseases in human and animal samples and drug screening *in vitro* and *in vivo*.

PAD-Beads are an ideal generic front-end separation technique that can be combined with a back-end protein-specific commercial or in-house ELISA detection to enable the specific detection of abnormal aggregated proteins. To date **PAD-Beads** have been demonstrated to work with β-amyloid, tau, α-synuclein, huntingtin and prion proteins*.

Abnormally folded and aggregated proteins can be separated *in vivo* in human disease, in animal models, in cell culture and *in vitro* using aggregated recombinant proteins. For example, **PAD-Beads** have been used to separate: abnormal β-amyloid and tau in Alzheimer's Disease in both human disease and in animal models; and abnormal prion protein in both sporadic and new variant Creutzfeldt-Jakob Disease (CJD), in Bovine Spongiform Encephalopathy (BSE) in cows, in Chronic Wasting Disease (CWD) in deer and in scrapie, including atypical scrapie in sheep. A form of the Seprion-PAD technology used in **PAD-Beads** has received USDA and EU approval for animal testing for prion disease (the Idexx Herdchek Test).

***PAD-Beads** are likely to work with other Protein Aggregation Diseases including: amylin in Diabetes Type II; crystalline in cataracts; antibody light chain, serum amyloid A, and β2-microglobulin in amyloid including primary and secondary systemic amyloidosis; and superoxide dismutase 1 in amyotrophic lateral sclerosis but these diseases remain to be tested.

II. Kit contents for 25 separations

CB 25 ml Capture Buffer
SR 10 ml Seprion-PAD reagent.
B 10 ml of **PAD-Beads** paramagnetic beads
WB1 25 ml of Wash buffer 1. Make up to 125 ml with distilled water prior to use.
WB2 25 ml of Wash buffer 2. Make up to 250 ml with distilled water prior to use

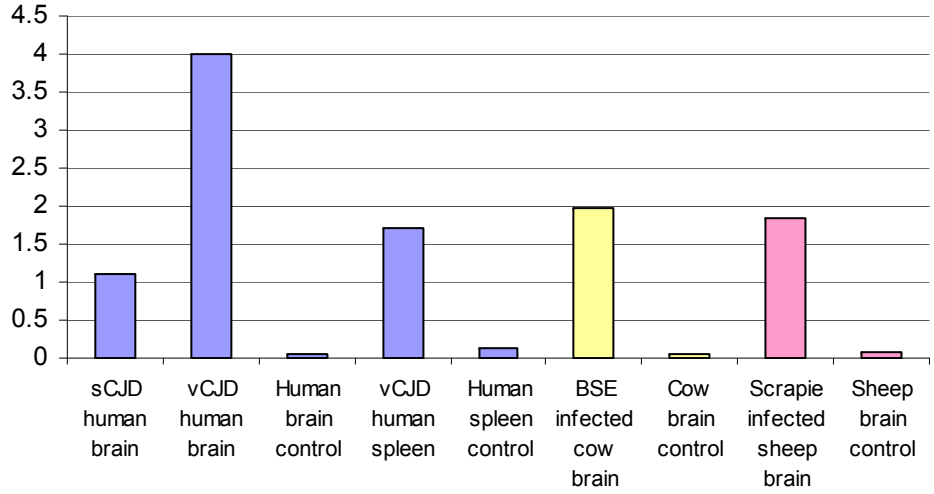
Store all reagents at 4-8°C

PAD-Beads for the study of Protein Aggregation Diseases

Example 3. A study of prion disease

Method
Prion diseases in various animal species were investigated i.e. sporadic Creutzfeldt-Jakob Disease (sCJD) and new variant Creutzfeldt-Jakob Disease (vCJD) in human, Bovine Spongiform Encephalopathy (BSE) in cows and scrapie in sheep. Brain and spleen samples were homogenised and 0.5 mg of each put through the PAD bead separation. After separation, the protein was eluted and put through an in-house ELISA assay for prion protein. Uninfected brain and spleen samples were assayed as controls.

Results
All of the brain and spleen samples infected with prion showed a high signal for abnormal prion protein (see graph below). All of the control samples were negative.



Conclusion
The **PAD-Beads** enable the separation of abnormal prion protein from brain and spleen from a range of infected species including man. The isolated abnormal prion protein can subsequently be detected by anti-prion ELISA.

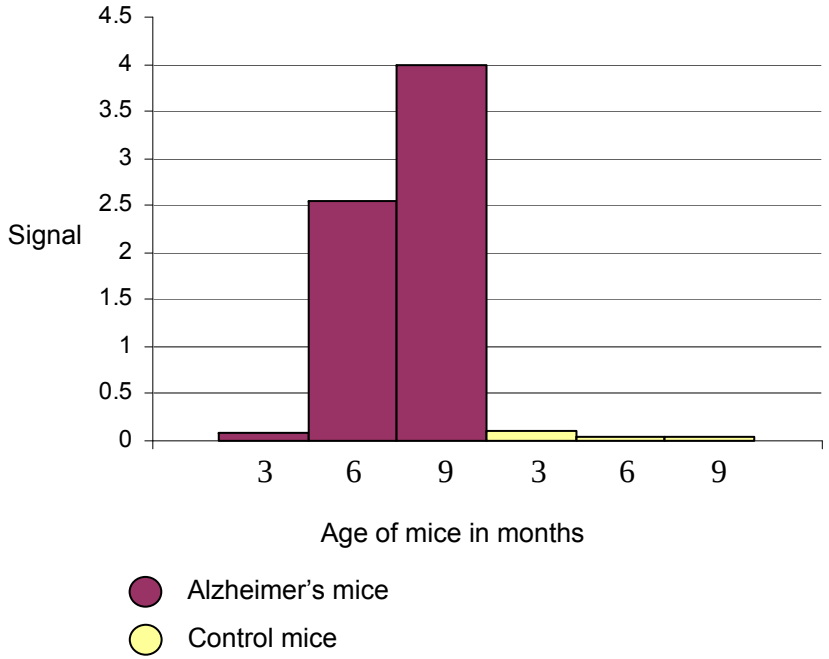
Example 2. A study of Alzheimer's mouse model

Method

A study was performed on a mouse model of Alzheimer's Disease. These mice develop amyloid plaques in the brain which can be seen histologically from 6 months onwards. Brains of mice at various ages were taken and homogenised. The abnormal β-amyloid protein was separated from the normal protein using **PAD-Beads** and after separation the eluted protein was investigated by ELISA in the Biosource 1-40 β-amyloid assay. Brains from normal mice were also assayed as controls.

Results

The results were plotted in a graph. The graph shows the mean data from 0.5 mg of brain from each of three mice at each age.



Conclusion

Using **PAD-Beads** it was possible to show that the amount of abnormal amyloid protein increased significantly from 3 to 6 months of age in the brains of the mouse model at which time pathological changes are know to begin to appear in the brain. No such increase and no abnormal amyloid was detected in the normal mouse brains.

PAD-Beads for the study of Protein Aggregation Diseases

III. Guidelines to sample preparation prior to using PAD-Beads.

PAD-Beads are a flexible approach for the separation of abnormal proteins present in a range of Protein Aggregation Diseases from a diverse number of tissues and bodily fluids. While the protocol for the separation using the kit is defined there is an element of trial and error for the sample preparation prior to use of the kit which depends on the protein and sample to be investigated. The following is supplied as a guideline for different samples and tissues and may prove useful in the preparation of samples prior to using **PAD-Beads**.

Liquid samples

Liquid samples such as plasma, serum and CSF can be used. The sample volume should be limited to 200µl. Prior to adding plasma to the **PAD-Beads** it may be beneficial to remove fibrinogen by heating at 56°C for 15 min followed by centrifugation at 20,000 x g for 5 min. The supernatant can then be used. It may be useful to use protease treatments to reduce any effect of the sample matrix on the capture. Trypsin or proteinase K can be included in the capture step at final concentrations of 50-250µg/ml. The effect of adding these proteases and the exact concentration to be used should be determined empirically as the effect of these reagents will vary with sample and disease types.

Solid tissue samples

Tissue samples such as brain should be first homogenised in water or 5-20% sucrose as a 10-25% homogenate. A ribolyser or hand held homogeniser may be used. No more than 25 mg of tissue should be used per separation reaction in a total volume of 200µl homogenate. It may be beneficial to add SDS to the homogenate to a final concentration of 0.1-1% (w/v) to help solubilise the tissue. Again, as with liquid samples it may be useful to use protease treatment to reduce any effect of the sample matrix on the capture (see above).

Soft tissue samples such as white cell samples, spleen and cell culture samples

It may be useful to treat these samples with DNAse 1 before PAD-Bead separation. Samples can be treated with 1% (v/v) Triton X-100, 0.1mM $MgCl_2$, 100µg/ml DNAse 1 for 30 min prior to use. No more than 200µl of treated sample should be used in the subsequent separation procedure. As with liquid samples (see above) it may be useful to use protease treatment to reduce any effect of the sample matrix on the capture.

Storage of samples prior to separation

All samples and tissues processed or unprocessed can be stored frozen until separation is performed. Prior to analysis defrost and mix the samples thoroughly.

IV. PAD-Beads Separation Protocol

Capture

For each separation, start with 200µl of sample that has been processed as described above (see the guidelines in section III). If the volume is less than 200µl make up the volume with distilled water.

1. To the 200µl of sample in a microtube add 200µl of Capture buffer, mix thoroughly and then add 400µl distilled water.
2. Add 100 µl PAD reagent and mix. Incubate for 10 min.
3. Add 100µl **PAD-Beads** (carefully resuspend the beads prior to use).
4. Shake by vibration (so that the beads do not settle) for 30 min at room temperature.
5. Capture the beads on a magnet and remove the liquid.
6. Add 1ml Wash buffer 1 to the beads and resuspend by vortexing.
7. Recapture the beads and wash twice with 1 ml Wash buffer 2.
8. Capture the beads and remove the liquid.
9. Remove the tubes from the magnet and pulse spin in a microfuge. Place back on the magnet and remove the last dregs of liquid.

Elution methods

Cold elution method (for subsequent analysis by ELISA)

The captured protein can be eluted and analysed by ELISA for the protein of interest.

1. Add 10-50 µl of 0.1 M NaOH, 0.1% Triton X-100, resuspend the beads and shake for 5 min.
2. Place the tubes on a magnet to capture the beads.
3. Still on the magnet, add the same volume of 0.1M HCl to neutralise the alkali and mix briefly by pipetting up and down.
4. Remove the liquid and analyse by ELISA. The eluted liquid can be mixed with the sample diluent supplied with most commercial or in-house ELISAs and tested directly.

Note. A higher molarity of NaOH may be used for elution and neutralised with an equivalent molarity of HCl, as long as the final eluate is compatible with the subsequent ELISA analysis. Similarly, a higher concentration of Triton may be used or other detergents such as sarkosyl or SDS may be tried.

Hot elution method (for Polyacrylamide gel electrophoresis [PAGE] or ELISA)

The captured protein can be eluted and analysed by PAGE. Proteins are generally denatured by boiling in gel loading buffer prior to PAGE analysis. In the same way the beads and the captured protein can be boiled in gel loading buffer, the beads separated by a magnet and the eluted proteins analysed by PAGE. Alternatively, instead of PAGE and depending on the ELISA used, it is often possible to elute the sample by boiling for 5 min in 25 µl 0.75% (w/v) SDS and analyse the eluate by ELISA as long as the sample is sufficiently diluted by the ELISA buffer used for capture. It may be helpful to supplement this buffer with 5% (w/v) BSA as a further precaution.

PAD-Beads for the study of Protein Aggregation Diseases

Examples of the use of PAD-Beads

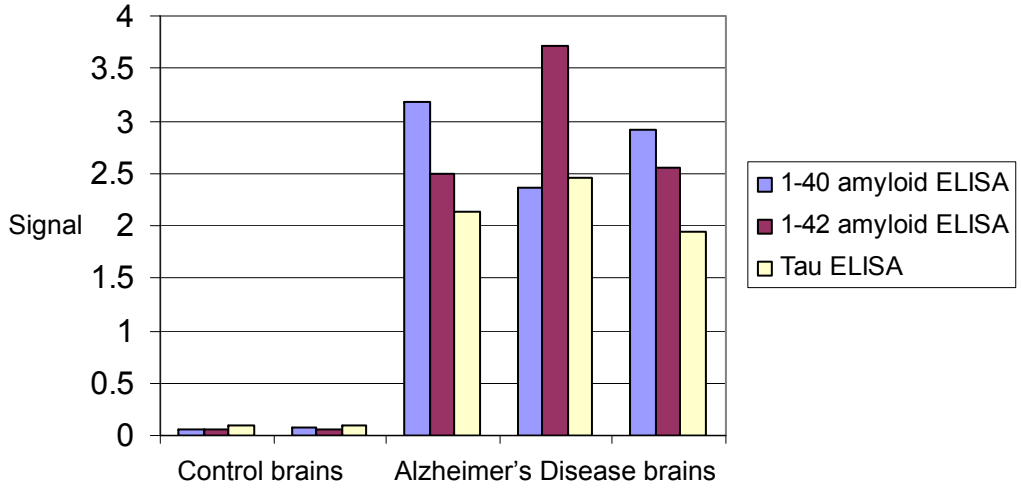
Example 1. A study of human Alzheimer's Disease

Method

Five brain samples, two normal and three confirmed Alzheimer's Disease, were examined for the presence of abnormal β-amyloid and abnormal tau protein. The brain samples were homogenised and the abnormal amyloid and tau proteins separated using **PAD-Beads**. After separation, the eluted protein was analysed for abnormal β-amyloid by both the Biosource 1-40 and the Biosource 1-42 β-amyloid ELISA and for abnormal tau protein by the Biosource Tau ELISA. 0.5mg of brain was investigated for amyloid and 1.5 mg of brain was investigated for tau.

Results

The brains from the confirmed Alzheimer's Disease patients had much higher levels of both the 1-40 and 1-42 abnormal β-amyloid protein and also of the abnormal tau protein compared to the age-matched control brains (see graph below).



Conclusion

Using the **PAD-Beads** to separate normal from abnormal protein it could be shown that the brains of the Alzheimer's Disease patients contained large amounts of both abnormal 1-40 and 1-42 β-amyloid and abnormal tau protein compared to the brains from age matched controls without Alzheimer's Disease.