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The WRC operates in terms of the Water Research Act (Act 34 of 1971) and its mandate is to support water research and development as well as the building of a sustainable water research capacity in South Africa.

TECHNICAL BRIEF

Water & health

Developing an EDC detecting system

A completed WRC study investigated the development of an endocrine disrupting compound (EDC) detection system using the ligand binding domains of the human receptor alpha and human androgen receptor.

Background

There is growing evidence that certain hormone active agents in the environment can disrupt chemical messengers (hormones) of the endocrine system. The putative hormone active agents, also known as endocrine disrupting compounds (EDCs), exert their deleterious effects on humans and wildlife by mimicking, blocking and disrupting the physiological functions of hormones.

Hormones exert their functions by interacting with their corresponding receptors in target cells to trigger responses and prompt normal biological functions, such as growth, development, behaviour and reproduction. Interference with the activities of hormones, such as is the case with EDCs, can lead to reversible or irreversible abnormal biological outcomes, including stunted growth, impairment of short-term memory, tubal pregnancy, low sperm count, reproductive failure, and damage of the immune system. it is clear that as researchers continue to look at the adverse effects caused by these hazardous compounds on humans and wildlife, they continue to find significant, often permanent, effects at remarkably low doses.

EDCs in the environment

EDCs have been widely reported to be present in very low concentrations in the environment, but their relatively high fast solubility causes these substances to bio-accumulate in fat deposits of organisms and animals higher up in the food chain, leading to significant physiological responses at these relatively low concentrations.

The hydrophobicity of EDCs, coupled with other chemical properties, has created unique challenges for environmental analytical chemists in developing techniques required for detecting and screening them. Several analytical techniques have been used. These methods frequently include soli phase extraction (SPE) followed by: high performance liquid chromatography (HPLC), liquid chromatography/mass spectrophotometry (LC/MS) or gas chromatography/mass spectrophotometry (GC/MS).

These techniques are, however, limited for general EDC monitoring due to relative high instrument costs, intensive labour and, in some instances, relatively poor sensitivity. In addition, high-end analytical procedures are usually specific for one single analyte only, or a limited class of structurally related compounds.

Alternative EDC detection

Affinity chromatography (AC) is a powerful chromatographic technique, which utilises the specific interaction between a biological molecule and a ligand (hormone receptor and hormone) to affect the specific binding and isolation of a ligand or substrate analogue.

During AC bio-specific and reversible interactions are used for the selective separation and purification of biological molecules from complex biological matrices. These systems are increasingly applied in the field of biotechnology due to the ability of the techniques to specifically bind and remove bio-molecules from complex mixtures.

However, despite their increased use, the technique still has some shortcomings, including the requirements for a large

column set-up and a longer diffusion path length, which in turn leads to a significant increase in the time required for the entire downstream processing from the introduction of the crude extract to the final purified product.

Membrane affinity chromatography (MAC) was introduced to overcome the major shortcomings of column affinity chromatography. Its introduction has significantly reduced the number of steps needed to obtain a pure product due to the specificity of the interaction between the stationary phase and the target bio-molecule, not withholding the larger surface area and shorter diffusion path length that the system offers.

With the above-mentioned advantages affinity membrane systems could serve as a powerful method for analytical detection, and possibly removal processes, for EDCs from the environment.

Estrogenic compounds and their detection

Estrogenic compounds are a class of EDCs which mimic or block the endogenous estrogen activity by binding to the ligand binding domain (hERαLBD) of estrogen receptors (ERs) in the endocrine system. exploiting the interaction between estrogen and its receptors, and using the chemical information obtained from this interaction, a more reliable and specific analytical functionalised affinity membrane system for the initial capture, concentration and qualitative detection of EDCs can be developed.

This method will be supplemental and used in conjunction with high-tech analytical techniques for detecting EDCs in the environment. a similar system can also be developed for the androgenic EDCs using the hARLBD. These affinity-based rapid detection systems will be a useful addition to the current 'toolbox for EDC monitoring and detection' under development by the WRC.

Rationale and aims of WRC study

The development of a 'dip stick system' was proposed based on the following principles. The ligand binding domain of the hERαLBD and the hARLBD are immobilised on a membrane contactor using non-covalent AC technology. The immobilised receptor Is exposed to the water containing low concentrations of estrogenic and/or androgenic compounds.

These compounds are bound to the corresponding receptor and concentrated on the contactor surface through the receptor ligand interaction. After activation – a mild increase in temperature – the hERαLBD or hARLBD estrogenic/androgenic compound-complex can be indicated using specific antibodies in an enzyme-linked immune-assay system.

The presence of estrogenic/androgenic compounds will be indicated by the development of a specific colour on the contactor 'strips'.

The main aims of the WRC-funded study were then to develop:

- A 'dip stick system' using the ligand binding system domain of the estrogen (hERαLBD) and androgen receptor (hARLBD) immobilised non-covalently on a hydrophobic membrane contactor matrix
- An enzyme-linked immunosorbent assay (ELISA)-based detection system for the qualitative indication of bound estrogenic compounds.

Results

Membrane fabrication

Synthetic polymeric membranes were fabricated using the immersion precipitation technique to manufacture nonporous planar and capillary membranes of reproducible physical and chemical composition. The surface chemistries of the membrane polymers were verified using photo acoustic FT-IR analysis.

Surface hydrophobicity was calculated using static and dynamic contact angle analysis for the planar and capillary membranes respectively. The candidate membranes chosen in this study had rough surfaces that were inherent to the fabrication conditions use.

Membrane surface roughness, however, was found to decease after surface modification in 5 mg/ml Pluronic F108. The membrane surface hydrophobicity was of the order PVDF > PSU > PEI.

Imidazole was used to activate and solubilise EDTA-dianhydride. This yielded a tetra dentate ligand with coordination sites on the octahedral system open for ligand attachment and a non-polar centre block available for hydrophobic surface interaction.

A CA/amylose mixture was successfully used as a casting dope for the fabrication of a CA/amylose functionalised membrane using the immersion precipitation technique. The membrane topography and morphology was studied

with the aim of the SEM technique, while the surface chemistry of the membrane was monitored with FT-IR.

Following the information obtained from the membrane morphology and surface chemistry the membrane could be used for affinity immobilisation of the specific bio-ligands MBP and MBP-hERαLBD fusion proteins. Membranes containing higher percentages of amylose were ductile, fragile and therefore could not be used as solid supports for further experimental analyses.

Therefore, in the present study, only a 2% amylose membrane was used for the immobilisation study. In future, however, higher percentages of amylose could be incorporated into the CA membrane but this will be followed by using some plasticiser to render the membrane more resilient to physical damage and deformation.

Receptor expression

The hERαLBD insert was confirmed to be present in the pET15b as shown via the restrict digestion performed on the isolated plasmid DNA. Analysis via SDS PAGE and Western Blot confirmed the hERαLBD was expressed and was present in the supernatants of both the media which contained additives (E2 and sucrose) and media which contained no additive.

Resuspended pellets contained hERαLBD, though most of the hERαLBD remained in the solution and thus in the supernatant.

The LBD of the AR, isolated and amplified via PCR, was successfully subcloned into the pTrc-His plasmid. The plasmid construct, designated AR-pTrcHis, was then used to transform TOP10 E.coli. expression of the histidine tagged ARLBD was confirmed via SDS PAGE and Western Blot analysis.

This study showed that MBP-hERαLBD ligated into pMalc2, and the MBP gene (MalE product) from pMalc2 ca be expressed in high yield using the E.coli expression system.

Receptor purification and antibody production

The hERαLBD and ARLBD were purified on the AKTA® protein purification system. the one-step affinity purification system with amylose as the solid phase was used for the effective purification of MBP- hERαLBD proteins.

Antibodies were raised against the estrogen bound and unbound forms of the hERαLBD. There is a degree of distinction between the E2 bound and unbound form of hERαLBD at higher serum dilutions. Sensitivity at lower serum dilutions is diminished and the primary antibodies are unable to distinguish between the two forms of hERαLBD.

Binding studies

The initial tests were carried out in polystyrene microtitre plates. The Pluronic-DMDDO, charged with hERαLBD, was coated onto the surface of the polystyrene microtitre plate. Two controls used in the experiment included wells that were coated with Pluronic F108 and uncoated wells.

A significantly higher binding of radioactive estrogen could be shown in the wells coated with Pluronic-DMDDo charged with hERαLBD when compared to that of the controls, indicating immobilisation of hERαLBD.

The second Pluronic-DMDDO experiment, charged with his_6-ARBLD, yielded similar results in that the wells charged with the protein were able to bind a significantly higher amount of radioligand compared to the controls. These experiments clearly indicate proof of concept as it is apparent that not only were the LBDs of the androgen and estrogen receptors immobilised, but the receptors retained bioactivity by binding to androgenic and estrogenic compounds in water solutions, respectively.

Prior to the CA/amylose flat-sheet membrane binding assay, the activity and binding of the recombinant protein, MBP-hERαLBD, onto the resin was tested using ^{3}HE2. For the determination of the E2-binding, a slurry of amylose resin was incubated overnight with extract containing MBP-hERαLBD.

The washed slurry was later incubated for three hours at room temperature with 300 µL of buffer A containing ^{3}HE2 working solution. The washing step was repeated and 4 ml of scintillation cocktail was added to the slurry. The mixtures were transferred into scintillation vials and later counted using a liquid scintillation counter.

Conclusions

The preliminary results conclusively demonstrated that the affinity immobilisation of estrogenic and androgenic compounds via the LBD of the human estrogen and androgen receptors on inert membrane contactors is possible and practically feasible. Although all the aims set out have been achieved for the human estrogen/androgen receptor LBD, significantly more work is needed to streamline and implement the practical implementation of the technology.

Once this has been completed, the technology can be field tested, initially in drinking water plants to monitor the long-term accumulation of EDCs. A more robust system will also be developed for surface waters. The system, although easy to operate, will have to be explained to users in the field via hands-on workshops.

Further reading:
The development of an endocrine disrupting compound detection system using the ligand binding domains of human estrogen receptor alpha (hERaLBD) and human androgen receptor (hARLBD) – proof of concept (**Report No. 1534/1/13).**
To order the report, contact Publications at Tel: (012) 330-0340, Email: orders@wrc.org.za or Visit: www.wrc.org.za to download a free copy.