

DETECTING ENDOCRINE DISRUPTING COMPOUNDS

THIS INVENTION relates to detecting endocrine disrupting
5 compounds. In particular the invention relates to a detection device for
detecting endocrine disrupting compounds and a method of detecting endocrine
disrupting compounds.

BACKGROUND OF THE INVENTION

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It is well known that certain hormone active agents in the
environment can disrupt chemical messengers (hormones) of the endocrine
system by sending erroneous signals or blocking legitimate signals. The
putative hormone active agents, also known as endocrine disrupting
15 compounds (EDCs), exert their deleterious effects on humans and wildlife by
mimicking, blocking and disrupting the physiological functions of hormones. The
Endocrine Society of America brought out a comprehensive report in 2009
presenting evidence that endocrine disruptors effect male and female
reproduction, breast development and cancer, prostate cancer,
20 neuroendocrinology, thyroid activity, metabolism, obesity and cardiovascular
endocrinology (Diamanti-Kandarakis et al., 2009). The report concludes that
results from animal models, human clinical observations, and epidemiological
studies supply overwhelming evidence that EDCs pose a significant concern to
public health.

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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
30 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.

5 Endocrine disrupting compounds can be categorized into three major groups: androgenic (compounds that mimic or block natural testosterone), thyroidal (compounds with a direct or indirect impact on the thyroid glands) and estrogenic (compounds that mimic or block natural estrogens). Despite the broad spectrum of EDCs, the estrogenic compounds
10 (ECs) are the most prominent and studied of these classes of compounds. This is in all likelihood due to the importance of ECs in cancer research, specifically related to female sexual development and disorders (McLachlan et al., 2006; Darbre and Charles, 2010; Fenton, 2006; Newbold et al., 2007). Estrogenic compounds are found in low doses in literally thousands of products, and
15 include compounds like diethylstilbestrol, bisphenol A, polybrominated diphenyl ethers and phthalates. Androgenic EDCs have also attracted much attention due to their effects on male virility and fertility (Bay, Asklund et al., 2006; Sharpe, 2006; Skakkebak et al., 2001). Endocrine disrupting compounds have been widely reported to be present in very low concentrations in the
20 environment, but their relatively high fat 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. Other relevant sources of EDCs are found in insecticides, herbicides, fungicides, plasticizers, plastics, resins and industrial chemicals
25 such as detergents (Diamanti-Kandarakis et al., 2009). 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 solid phase extraction (SPE) followed by:
30 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.

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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 (Cuatrecasas, 1970). 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 technique to specifically bind and remove bio-molecules from complex mixtures. Typical affinity systems consist of two distinct parts: the mobile phase, which carries the biological molecule to be separated, and the solid phase, which is usually modified to carry the affinity ligands. Notwithstanding the fact that these systems are widely used, they still have 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 (AMS) could serve as a powerful method for analytical detection, and possibly removal processes, for EDCs from the environment.

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Estrogenic compounds are a class of EDCs which mimic or block the endogenous estrogen activity by binding to the ligand binding domain

(hER α LBD) 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 functionalized affinity membrane system for the initial capture, concentration and qualitative detection of ECs can be developed. This method will be supplemental and used in conjunction with high technology analytical techniques such as HPLC, GC/MS and LC/MS for detecting ECs in the environment. A similar system can also be used for the androgenic EDCs using the hARLBD.

The inventors identified a new detection device, which is rapid and efficient in monitoring and detecting endocrine disrupting compounds.

References in this specification to the term "sex hormone receptors" specifically refer to estrogen and androgen receptors.

In this specification the following abbreviations will be used:

	³ HE2	2, 4, 6, 7 ³ H 17 β –estradiol
20	AC	Affinity chromatography
	AMS	Affinity membrane system
	AR	Androgen receptor
	CA	Cellulose acetate
	CPM	Counts per minute
25	DMDDO	Dicarboxymethyl-3,6-diazaoctanedioate
	DMF	Dimethyl formamide
	DMSO	Dimethyl sulphoxide
	E2	17 β – estradiol
	EC	Estrogenic compound
30	EDC	Endocrine disrupting compound
	EDTA	Ethyl diamine tetraacetic acid
	ELISA	Enzyme linked immunosorbent assay

	ELRA	Enzyme linked receptor assay
	ER	Estrogen receptor
	ERE	Estrogen response element
	FT-IR	Fourier Transformed infrared spectroscopy
5	GC	Gas chromatography
	GC/MS	Gas chromatography/Mass spectrophotometry
	HF	Hollow fibre
	HFF	Hollow fine fibre
	his ₆	Hexahistidine
10	HPLC	High performance liquid chromatography
	HRP	Horseradish peroxidase
	IMAC	Immobilized metal ion affinity chromatography
	LBD	Ligand binding domain
	LC/MS	Liquid chromatography/Mass spectrophotometry
15	LLD	Lowest limit of detection
	MAC	Membrane affinity chromatography
	MBP	Maltose binding protein
	MS	Mass spectrophotometry
	NMR	Nuclear magnetic resonance
20	PCR	Polymerase chain reaction
	PDMDDO	Pluronic- <i>N,N</i> -dicarboxymethyl-3,6-diazaoctanedioate
	PEI	Polyether imide
	PIXES	Proton induced x-ray emission spectrophotometry
	PS	Polystyrene
25	PSU	Polysulphone
	PVDF	Polyvinylidene fluoride
	SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
	SEM	Scanning electron microscopy
	SPE	Solid phase extraction
30	UF	Ultrafiltration

In this specification reference will be made to the following documents:

- BAY, K., ASKLUND, C., SKAKKEBAEK, N.E. and ANDERSSON, A.M., 2006. Testicular dysgenesis syndrome: possible role of endocrine disrupters. *Best Practice & Research in Clinical Endocrinology & Metabolism*, **20**(1), 77-90.
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SUMMARY OF THE INVENTION

According to one embodiment of the invention, there is provided a detection device for detecting endocrine disrupting compounds, which includes
10 a support structure; and
ligand binding domains (LBD) of at least one sex hormone receptor, immobilised on the support structure.

The detection device may include a linker molecule between the
15 support structure and the ligand binding domain.

The endocrine disrupting compounds may be in the form of any one or more of androgenic and estrogenic compounds.

20 The sex hormone receptor may be in the form of any one or more of an estrogen receptor and an androgen receptor. Specifically the sex hormone receptor may be in the form of any one or more of a human estrogen receptor and a human androgen receptor, having ligand binding domains hER α LBD and hARLBD respectively. The ligand binding domain may include a
25 peptide tag and be in the form of his₆ – hER α LBD or his₆ – hARLBD. Alternatively the ligand binding domain may be expressed as a fusion protein and be in the form of maltose binding protein – hER α LBD (MBP – hER α LBD).

The ligand binding domain of the sex hormone receptor, may be
30 prepared by cloning the genes encoding the ligand binding domain and expressing the genes in a host. Preferably the host may be *Escherichia coli*.

The support structure may be in the form of a membrane. The support structure may be in the form of a membrane contactor (strip matrix).

In one embodiment the membrane contactor may be in the form of
5 a cellulose acetate hybrid membrane, specifically an affinity cellulose acetate-amylose hybrid membrane. In such embodiment the linker molecule may be in the form of maltose binding protein.

In another embodiment the support structure may be in the form of
10 an inert membrane. The inert membrane may be in the form of a synthetic polymeric membrane. The synthetic polymeric membrane may be fabricated using the immersion precipitation technique. The synthetic polymeric membrane may be planar and nonporous. The synthetic polymeric membrane may be cast from any one or more of polysulphone (PSU), polyether imide (PEI)
15 and polyvinylidene fluoride (PVDF) solutions.

In such embodiment the linker molecule may be in the form of a modified poloxamer. The poloxamer may be in the form of a difunctional block copolymer surfactant terminating in primary hydroxyl groups. The poloxamer
20 may be in the form of poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (hereafter referred to as Pluronic F108, also known as PEG-PPG-PEG).

The modified Pluronic F108 may be in the form of Pluronic F108 of
25 which the hydroxyl end groups was modified to a metal chelating moiety which can specifically bind Ni-ions and serve as an immobilised metal affinity chromatography matrix and provides a metal affinity immobilised receptor ligand binding domain. Specifically an ethylene diamine tetraacetic acid dianhydride may be coupled to the terminal hydroxyl end groups of Pluronic F108 via a two-
30 step reaction, to create the new metal affinity ligand, Pluronic-N,N dicarboxymethyl-3,6-diazaoctanedioate (Pluronic-DMDDO).

Another aspect of the invention relates to a method of detecting endocrine disrupting compounds, which includes

- contacting a detection device as described with a sample to be tested, such that if the sample contains endocrine disrupting compounds, the compounds will bind to the ligand binding domains; and
- performing a colorimetric assay to indicate bound endocrine disrupting compounds.

The sample to be tested may be in the form of a water sample.

Contacting the detection device with the sample, may be by way of submerging the detection device in the sample, dropping the sample onto the detection device, dipping the detection device in the sample or the like.

In one embodiment performing the colorimetric assay may include the steps of

- saturating the receptors with EDCs, upon which the receptors change conformation and are activated;

- adding monoclonal antibodies, raised against the activated receptors, and coupled to enzymes, allowing the antibody-enzyme complex to bind to the activated bound receptor;

- observing the bound enzymes, which indicates the presence of EDCs in the sample.

In this embodiment the enzyme may be in the form of an enzyme which produces a coloured, fluorimetric or luminescent derivative of the activated bound receptor. The enzyme may be in the form of horse radish peroxide.

In another embodiment performing the colorimetric assay may include the steps of

- adding enzyme labelled steroid ligands for the specific receptors; and

determining the degree of receptor saturation.

In yet a further embodiment performing the colorimetric assay may include the steps of

5 adding heat shock proteins, which binds and activates bound receptors;
and

 adding antibodies against the heat shock proteins to indicate bound receptors and thus endocrine disrupting compound binding.

10 The invention will now be described , by way of example only with reference to the following representation(s):

DRAWING(S)

In the drawing(s):

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Figure 1 shows a schematic representation of the immobilized steroid ligand binding domain for the affinity capture of EDCs in drinking water;

Figure 2 shows a schematic representation of a method of detection of bound EDCs.

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EMBODIMENT OF THE INVENTION

In Figure 1 a schematic representation of a detection device for detecting endocrine disrupting compounds is shown. The detection device
25 includes a support structure in the form of a membrane contactor and ligand binding domains (LBDs) of at least one sex hormone receptor, immobilised on the support structure. The detection device further includes a linker molecule, in the form of modified PluronicMDDO between the support structure and the ligand binding domain.

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The detection device works on the following principles: The ligand binding domain of the hER α LBD and the hARLBD are immobilised on a

membrane contactor matrix using noncovalent 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.

In Figure 2 a method of detecting endocrine disrupting compounds is shown. The first step of this method entails contacting a detection device as described with a sample to be tested, such that if the sample contains endocrine disrupting compounds, the compounds will bind to the ligand binding domains. The second step is to perform a colorimetric assay to indicate bound endocrine disrupting compounds.

As shown in Figure 2, after activation, a mild increase in temperature, the hER α LBD- or hARLBD estrogenic/androgenic compound-complex can be indicated using specific antibodies in an enzyme linked immuno-assay system. The presence of estrogenic/androgenic compounds will be indicated by the development of a specific colour on the contactor "strips".

Methodology

Membrane Fabrication

Planar nonporous membranes were cast from appropriate solutions, polysulphone (PSU), polyether imide (PEI) and polyvinylidene fluoride (PVDF). The solutions were degassed before use to cast 200 μ m planar membranes. Nonporous hollow fibre (HF) and hollow fine fibre (HFF) membranes and externally unskinned ultrafiltration (UF) membranes were produced by the phase inversion technique using a dry-wet spinning process available at the Institute for Polymer Science at the University of Stellenbosch.

An amylose functionalized cellulose acetate (CA) membrane was fabricated from a solution of CA and amylose in DMSO via immersion precipitation. The casting mixture was then used to cast flatsheet membranes by immersion precipitation using water. The membranes were thoroughly washed with water and stored in a sodium azide solution until required. The surface membrane topography, thickness and functional groups modification were determined using SEM and FT-IR respectively.

A Pluronic® F108 modified linker was synthesized as follows: The terminal hydroxy groups of Pluronic were modified in a two-step reaction to yield the tetra dentate DMDDO type ligand at the hydroxyl terminals of Pluronic. This reaction was carried out by dissolving a ten-fold excess of the EDTA dianhydride and imidazole dry DMF after which Pluronic F108 was added and reacted for 8 h at 40°C. Methanol was subsequently added and reacted for another 8 h after which the DMF was removed *in vacuo*. The residue was treated with toluene to selectively dissolve ligand-modified-Pluronic from the DMDDO by-product. The final product was characterised mainly by nuclear magnetic resonance (NMR) with the aid of model ligands based on mono and diethylene glycol (Govender et al., 2006).

Receptor Expression

The introduction of a peptide tag, or even low molecular weight proteins, to the N- or C-terminus of proteins has become a convenient and facile method for protein purification via affinity chromatography. Examples of tags include polyhistidine tags and maltose to produce fusion proteins. As the principle of non-covalent immobilization of the truncated steroid receptors forms the basis for the test strip envisaged in this project, the histidine tag allows for the purification of the receptors and a method for non-covalent immobilization following the principle of IMAC. Thus the ligand binding domain of the steroid receptors had to be subcloned into a suitable vector encoding for a histidine tag,

the plasmid construct transformed into a suitable host organism and finally expressed as histidine tagged fusion proteins.

The gene encoding for a hexahistidine tagged hER α LBD, subcloned into pET15b (a plasmid that encodes for an N-terminal hexahistidine tag), was obtained from the University of Illinois at Urbana-Champaign. The hER α LBD protein was expressed in *Escherichia coli* BL21 (DE3) pLysS. The initial yield of the soluble protein compared to the insoluble protein, was insufficient. Experiments to improve the solubilisation of the hER α LBD protein prior to purification were performed. These included the use of the detergent Nonidet P-40 (NP-40), which increases protein solubility, during cell lysis. In addition, 17 β -estradiol (E2) and sucrose were included during the expression experiments as previous studies showed that these additives increased the yield of soluble vs. insoluble protein. Protein detection methods (sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) and Western Blot analysis) showed that the increase in soluble hER α LBD protein was achieved in the presence of 10 μ M E2 (expression results in the presence of E2 showed a higher soluble protein when compared to the expression without E2).

Using the full length AR gene the truncated human androgen receptor ligand binding domain, hARLBD, was amplified via polymerase chain reaction (PCR) and subcloned into pTrcHis, a plasmid encoding for an N-terminal hexahistidine tag. The construct was designated AR-pTrcHis. The construct was subsequently used to transform TOP10 *E. coli*. Expression and purification of the hARLBD were identical to that of the hER α LBD with the exception of the absence of 17 β -estradiol.

In this study the targeted protein hER α LBD was expressed as a fusion protein to maltose binding protein (MBP). Maltose binding protein, being the main supplier of carbon to gram-negative bacteria, is known to have a great affinity for maltose and, in the absence of the maltose, has a great affinity for amylose. The affinity chromatographic column used in this study was set up

using amylose resin. Purification of the recombinant protein was possible, since these proteins will couple (using the MBP) to the amylose column via affinity interaction. The gene was ligated into a plasmid expression vector pMalc2 and transformed into *E. coli* TB1 cells for expression. The hER α LBD was expressed as a fusion protein to fusion partner MBP, a MalE gene of molecular mass 40.6 kDa.

Receptor purification and antibody production against fusion receptors

Preliminary purification of the hER α LBD protein with the use of a prepacked nickel agarose column (His SpinTrap, GE Healthcare) showed efficient binding of the histidine tagged hER α LBD protein to the resin. The protein was subsequently purified on a preparative AKTA® system. The hARLBD was purified under identical conditions to that of the hER α LBD. The MBP-hER α LBD fusion proteins and the MBP proteins were purified on an amylose column by affinity chromatography. The coupled fusion protein was eluted from the affinity column by including 10 mM of maltose in the elution buffer.

The purified hER α LBD, saturated with E2, was then inoculated into a rabbit in order to generate polyclonal antibodies against the E2-bound form of hER α LBD. Blood samples were drawn from the rabbit on days 0 and 28. The blood samples were stored at 4°C and allowed to coagulate after which they were centrifuged, separating the serum from the coagulant. The serum was used as the source of antibodies.

Binding Studies

For estrogen binding studies, PVDF membranes and polystyrene microtitre plates (Immunosorp Nunc®) were used as a scaffold for the Pluronic-DMDDO. Microtitre plates, in addition to the hydrophobic nature of the

polystyrene polymer from which they are constructed, are experimentally more practical, easier to handle and allow for greater throughput. Wells of a polystyrene plate were incubated with a solution of Pluronic-DMDDO. In addition, wells were incubated with a solution of Pluronic F108. The Pluronic-DMDDO wells were charged with Ni^{2+} . A purified hER α LBD solution (that had been dialysed overnight against a PBS buffer in order to remove E2 from the ligand binding domain (LBD) binding sites) was then added to the wells coated with modified and unmodified Pluronic. In addition, uncoated wells were coated with the purified hER α LBD solution. The wells were incubated with the hER α LBD solution. A working solution of 2, 4, 6, 7 ^3H -estradiol ($^3\text{HE2}$) was then pipetted into each well that contained either modified or unmodified Pluronic, or hER α LBD. The tritiated estradiol working solution was also applied to polystyrene wells that contained no additives. Wells were again washed with distilled water to remove excess radioactively labelled estradiol. Elution buffer (as per wash buffer used in purification procedure containing 500 mM imidazole) was then applied to each well. A volume of 80 μL of each sample was then transferred to individual scintillation tubes. Radioactivity of each sample was then measured via a scintillation counter (Tri-Carb®, 1900A) as counts per minute (CPM) over a period of 5 minutes. A second experiment was conducted using the his $_6$ -ARLBD and tritiated testosterone. The experimental parameters were otherwise exactly the same as in the hER α LBD/ $^3\text{HE2}$ experiment.

Maltose binding protein-hER α LBD fusion protein was immobilized to an amylose functionalized membrane with the amylose present in the membrane surface offering the binding sites for the MBP-hER α LBD immobilization since MBP has an affinity for amylose. The application of the membrane system for the selective recovery of estradiol from solution is also included herein.

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Results and discussion

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 add rough surfaces that were inherent to the fabrication conditions use. Membrane surface roughness was, however, found to decrease 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. The ligand Pluronic-DMDDO was characterised using ^{13}C NMR spectroscopy and is soluble in water organic solvents and can be stored indefinitely, either in solution or as a desiccate.

A CA/amylose mixture was successfully used as a casting dope for the fabrication of a CA/amylose functionalized membrane using the immersion precipitation technique. The membrane topography and morphology was studied with the aid 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 immobilization of the specific bio-ligands MBP and MBP-hER α LBD 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 immobilization study. In future, however, higher percentages of amylose could be incorporated into the CA membrane but this

will be followed by using some plasticizer to render the membrane more resilient to physical damage and deformation.

Receptor Expression

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The hER α LBD 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 hER α LBD 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 hER α LBD, though most of the hER α LBD remained in solution and thus in the supernatant.

The LBD of the AR, isolated and amplified via PCR, was successfully subcloned into the pTrcHis 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-hER α LBD ligated into pMalc2, and the MBP gene (MalE product) from pMalc2 can be expressed in high yield using the *E. coli* expression system. Results obtained from SDS PAGE and Western Blot analyses of the two respective cell lysates. A band corresponding to an apparent molecular mass of 66 KDa was in good agreement with the molecular mass of MBP-hER α LBD reported in literature.

Receptor purification and antibody production against fusion receptors

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

proteins. Antibodies were raised against the estrogen bound and unbound forms of the hER α LBD. There is a degree of distinction between the E2 bound and unbound form of hER α LBD at higher serum dilutions ($>10^{-2}$). Sensitivity at lower serum dilutions is diminished (between 10^{-1} and 10^{-2}) and the primary
5 antibodies are unable to distinguish between the two forms of hER α LBD.

Binding Studies

The initial tests were carried out in polystyrene microtitre plates.
10 The Pluronic-DMDDO, charged with hER α LBD, 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 hER α LBD when compared to that of the controls
15 (wells coated with Pluronic F108 and only and uncoated wells), indicating immobilization of hER α LBD. The second Pluronic-DMDDO experiment, charged with his₆-ARLBD, 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
20 apparent that not only were the LBDs of the androgen and estrogen receptors immobilized, 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
25 activity and binding of the recombinant protein, MBP-hER α LBD, onto the resin was tested using $^3\text{HE2}$. For the determination of the E2-binding, a slurry of amylose resin was incubated overnight with extract containing MBP-hER α LBD. The washed slurry was later incubated for 3 h at room temperature with 300 μL of buffer A containing $^3\text{HE2}$ working solution. The washing step was repeated
30 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

5 The inventors believe that the invention provides a new detection device for detecting endocrine disrupting compounds and that they have 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.

CLAIMS

1. A detection device for detecting endocrine disrupting compounds,
5 which includes
a support structure; and
ligand binding domains (LBD) of at least one sex hormone receptor,
immobilised on the support structure.
- 10 2. The detection device as claimed in claim 1, in which the detection
device includes a linker molecule between the support structure and the ligand
binding domains, for immobilising the LBD's on the support structure.
3. The detection device as claimed in claim 1, in which the endocrine
15 disrupting compounds are in the form of any one or both of androgenic and
estrogenic compounds.
4. The detection device as claimed in claim 3, in which the sex
hormone receptor is in the form of any one or both of an estrogen receptor and
20 an androgen receptor.
5. The detection device as claimed in claim 4, in which the sex
hormone receptor is in the form of any one or both of a human estrogen
receptor and a human androgen receptor, having ligand binding domains
25 hER α LBD and hARLBD respectively.
6. The detection device as claimed in claim 5, in which the ligand
binding domains include a peptide tag and is in the form of any one of his₆ –
hER α LBD and his₆ – hARLBD.
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7. The detection device as claimed in claim 5, in which the ligand
binding domain hER α LBD is expressed as a fusion protein and is in the form of
maltose binding protein – hER α LBD (MBP – hER α LBD).

8. The detection device as claimed in claim 1, in which the ligand binding domains of the at least one sex hormone receptor, is prepared by cloning genes encoding the ligand binding domain and expressing the genes in a host.

9. The detection device as claimed in claim 8, in which the host is *Escherichia coli*.

10. The detection device as claimed in claim 2, in which the support structure is in the form of a membrane.

11. The detection device as claimed in claim 2, in which the support structure is in the form of a membrane contactor (strip matrix).

12. The detection device as claimed in claim 11, in which the membrane contactor is in the form of a cellulose acetate hybrid membrane.

13. The detection device as claimed in claim 12, in which the cellulose acetate hybrid membrane is in the form of an affinity cellulose acetate-amylose hybrid membrane.

14. The detection device as claimed in claim 13, in which the linker molecule is in the form of maltose binding protein.

15. The detection device as claimed in claim 10, in which the support structure is in the form of an inert membrane.

16. The detection device as claimed in claim 15, in which the inert membrane is in the form of a synthetic polymeric membrane.

17. The detection device as claimed in claim 16, in which the synthetic polymeric membrane is fabricated using an immersion precipitation technique.

18. The detection device as claimed in claim 16, in which the synthetic
5 polymeric membrane is planar and nonporous.

19. The detection device as claimed in claim 16, in which the synthetic polymeric membrane is cast from any one or more of polysulphone (PSU), polyether imide (PEI) and polyvinylidene fluoride (PVDF) solutions.
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20. The detection device as claimed in claim 19, in which the linker molecule is in the form of a modified poloxamer.

21. The detection device as claimed in claim 20, in which the
15 poloxamer is in the form of a difunctional block copolymer surfactant terminating in primary hydroxyl groups.

22. The detection device as claimed in claim 21, in which the poloxamer is in the form of poly(ethylene glycol)-*block*-poly(propylene glycol)-
20 *block*-poly(ethylene glycol) (referred to as Pluronic F108).

23. The detection device as claimed in claim 22, in which the modified Pluronic F108 is in the form of Pluronic F108 of which the hydroxyl end groups are modified to a metal chelating moiety which can specifically bind Ni-ions and
25 serve as an immobilised metal affinity chromatography matrix and provides a metal affinity immobilised receptor ligand binding domain.

24. The detection device as claimed in claim 23, in which an ethylene diamine tetraacetic acid dianhydride is coupled to the terminal hydroxyl end
30 groups of Pluronic F108 via a two-step reaction, to create a new metal affinity ligand, Pluronic-N,N dicarboxymethyl-3,6-diazaoctanedioate (Pluronic-DMDDO).

25. A method of detecting endocrine disrupting compounds, which includes

contacting a detection device as claimed in any of claims 1 to 24 with a sample to be tested, such that if the sample contains endocrine disrupting compounds, the compounds will bind to the ligand binding domains; and

performing a colorimetric assay to indicate bound endocrine disrupting compounds.

26. The method of detecting endocrine disrupting compounds as claimed in claim 25, in which the sample to be tested is in the form of a water sample.

27. The method of detecting endocrine disrupting compounds as claimed in claim 26, in which contacting the detection device with the sample, is by way of any one of: submerging the detection device in the sample, dropping the sample onto the detection device and dipping the detection device in the sample.

28. The method of detecting endocrine disrupting compounds as claimed in claim 26, in which performing the colorimetric assay includes the steps of

saturating the ligand binding domains with EDCs, upon which the ligand binding domains change conformation and are activated forming activated EDC- ligand binding domain complexes;

adding monoclonal antibodies, raised against the activated EDC-ligand binding domain complexes, and allowing the monoclonal antibodies to bind to the activated EDC-ligand binding domain complexes,

using the monoclonal antibodies in an enzyme linked immuno-assay; and observing bound enzymes, which indicates the presence of EDCs in the

sample.

29. The method of detecting endocrine disrupting compounds as claimed in claim 28, in which the enzyme is in the form of an enzyme which produces any of a coloured, fluorimetric and luminescent derivative of the activated bound receptor.

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30. The method of detecting endocrine disrupting compounds as claimed in claim 29, in which the enzyme is in the form of horseradish peroxide.

31. The method of detecting endocrine disrupting compounds as claimed in claim 26, in which performing the colorimetric assay includes the steps of

adding enzyme labelled steroid ligands for the specific receptors; and
determining the degree of receptor saturation.

15 32. The method of detecting endocrine disrupting compounds as claimed in claim 26, in which performing the colorimetric assay includes the steps of

adding heat shock proteins, which binds and activates bound receptors;
and

20 adding antibodies against the heat shock proteins to indicate bound receptors and thus endocrine disrupting compound binding.

33. A detection device for detecting endocrine disrupting compounds as claimed in claim 1, substantially as herein described and illustrated.

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34. A method of detecting endocrine disrupting compounds as claimed in claim 25, substantially as herein described and illustrated.

35. A new detection device for detecting endocrine disrupting compounds, and a new method of detecting endocrine disrupting compounds, substantially as herein described.

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