2.0 Materials and Methods

tudy included: Sigma, USA; Fluka Chemika, from BDH Ltd., England; Merck, Republic of

antibodies IgG, IgA and IgM used in this study mones used in this study are from Sigma Co., rone, 4-androstene-3, 17-dione, adrenaline, oxyprogesterone, testosterone, noradrenaline, all ept for angiotensin II which was kept at 4 °C. β- dark container since it is air and light sensitive. Solvents used for hormones stock solution are listed in Table 2.1. Structures of hormones are showed in Appendix 3.

Table 2.1: Solvents used for hormone stock solutions

Hormones	Stock Solution (mg/ml)	Solvent Used	Vol. (µl) of stock soln. Per 10ml medium	Final conc. (µg/ml)
Adrenaline	5	PBS	100	50
Aldosterone	5	Absolute Ethanol	100	50
4-Androstene-3,17-dione	5	Absolute Ethanol	100	50
Angiotensin II	5	PBS	100	50
β-Estradiol	5	Absolute Ethanol	100	50
Hydro-Cortisone	5	Absolute Ethanol	100	50
Progesterone	5	Absolute Ethanol	100	50
Hydroxy-Progesterone	5	Absolute Ethanol	100	50
Noradrenaline	5	PBS	100	50
Testosterone	5	Absolute Ethanol	100	50

2.1 Samples

Normal human sera

Samples were collected from Kluang and Segamat, Johor Bahru, Malaysia, from healthy adult donors. All the samples were properly labeled with name, sex and age. The sera were then stored at -20 °C until they were tested for IgG, IgA and IgM.

Atherosclerosis patients sera

Samples from atherosclerotic patients were collected from the Cardiology ward of University Hospital, Kuala Lumpur (UHKL). The sera were stored at -20 °C until further use. The sera were tested for IgG, IgA and IgM. These samples are subsequently referred to as "cardiac serum".

Cord blood

Cord Blood Samples were collected from the labour ward of UHKL. Blood was drained from the umbilical cord into heparinised bottles as soon as possible after birth and subjected to lymphocyte separation within 24 hours at room temperature. The sera were tested for IgG antibodies.

Cerebrospinal fluid

Cerebrospinal fluid samples were collected from the Clinical Chemistry Laboratory of UHKL. The samples were tested for IgG antibodies.

2.2 Procedure for standard ELISA

1. Coating
2. Blocking
3. Serum Incubation
4. Conjugate Reaction
5. Substrate Reaction
6. Stopping Reaction
7. OD reading

Coating

Fifty microliters volumes of antigen at 50 μg/ml in absolute ethanol (Ajax Chemicals, Australia) were dispensed into microtiter plate wells (NUNC Immuno MaxiSorp, Denmark). Absolute ethanol alone was applied to wells to serve as antigen negative controls. Incubation was overnight at 4 °C, with the plate being uncovered for evaporation to occur.

Blocking

After overnight incubation at 4 °C, the plate was dispensed with 100 μl/well of 1% bovine serum albumin (BSA) / phosphate buffered saline (PBS) and incubated for 2 hours at room temperature (RT).

Serum incubation

After 2 hours incubation, the plates were washed 2 times with PBS washing solution, pH 7.2. Fifty microlitres per well of serum diluted 1:100 in BSA/PBS were

added and allowed to react for 2 hours at RT. All the sera were tested in duplicate The plates were washed 3 times with PBS washing solution.

Conjugate reaction

Horseradish peroxidase conjugated rabbit anti-human Ig (Dako Laboratories. Denmark) prepared at 1:1000 dilution in BSA/PBS. Then 50 µl of this diluted Ig was dispensed into each well and incubated for 2 hours at RT.

Substrate reaction

The plates were washed 4 times with PBS before 50 µl/well of the enzyme substrate *o*-phenylenediamine (10 mg/ml) with 30% H_2O_2 in citrate phosphate buffer, pH 5.6 was added.

Stopping reaction

The calorimetric reaction was stopped by the addition of 50 µl/well of 4M H_2SO_4. The optical absorbance (OD) was determined at 490nm / 630nm with an ELISA microplate reader (MRX Dynex technologies).

A serum was assigned positive when the difference (delta absorbance, dOD) between average OD of the antigen-positive and antigen-negative wells was ≥ 0.2. A dOD 0.2 - 0.3 was considered weakly positive (±), between 0.3 - 0.5, positive (+) and > 0.5, strongly positive (++) (Figure 2.1).

NOTE: Details of the solutions and buffers used in ELISA are in Appendix 4.

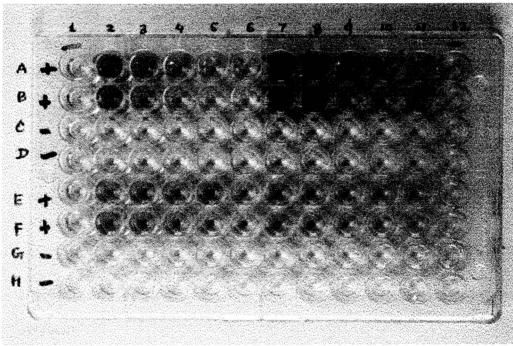

Figure 2.1: ELISA plate showing final calorimetric reaction

Rows: Rows A, B, E and F are coated with antigens (in duplicates) and are known as positive wells, whereas C, D, G and H contains no antigens (in duplicates) and are negative wells.

Columns: Column 2 (A), 2 (B), 2 (C) and 2 (D) were reacted with the same serum. The rest of the wells follow this pattern. Column 1 and 12 are blanks, i.e. they were inactivated with serum diluent.

2.3 Peptide Synthesis of the Apolipoprotein E Precursor using the Multipin Peptide Synthesis method

The chemicals used for the peptide synthesis procedure included N, N - dimethylformamide (DMF) (Sigma Co., USA), piperidine (Sigma Co., USA), acetic anhydride (Merck, Republic of Germany), N-ethyldiisopropylamine (Sigma Co., USA), trifluoroacetic acid (TFA) (Sigma Co., USA), ethanedithiol (Sigma Co., USA), anisole (Sigma Co., USA), 1-hydroxy-benzotriazole (HOBt) (Chiron technologies, Australia), and diisopropylcarbodiimide (DIC) (Millipore, USA).

Additional chemicals used for the (modified) ELISA procedure included, bovine serum albumin (BSA), Tween-20, sodium azide, rabbit serum, sodium caseinate.

MacCartney bottles, cylindrical jars and polypropylene microtiter trays used during peptide synthesis were thoroughly rinsed in water after a soak in Teepol / Chlorox and subsequently washed three times in distilled water and oven dried before being used.

Many of the solvents and reagents used in peptide synthesis are toxic and many are flammable and /or corrosive and are potentially hazardous. Skin and eye contact were avoided. Care was taken to wear gloves and safety lab coats at all times. All the synthetic steps were performed in a fume cupboard.

Synthesis was performed on the heads of polyethylene pins (crowns). Coupling reactions were performed in the wells of polypropylene microtiter trays, whereas washes or common reactions such as Fmoc-deprotection, was performed in baths.

Peptide Synthesis Procedure

1. Selection of synthesis regions and generation of the synthesis schedule
2. Pre-synthesis preparations
3. Weighing amino acids and activating chemicals
4. N, N- Dimethylformamide (DMF) filtration
5. F-moc deprotection of pins
6. Coupling the N- α - F-moc protected amino acids
7. Washing of synthesized pins
8. Acetylation of terminal amino groups
9. Side-chain deprotection

2.3.1 Chemistry of Peptide Synthesis

All amino acids recommended for the Multipin peptide Synthesis kit have their alpha- amino acid group protected with the 9-fluorenylmethyloxycarbonyl (F-moc) group. The following groups protect the amino acid side chains:

a) t-butyl ether (Bu^t) for serine, threonine and tyrosine

b) t-butyl ester (Obu^t) for aspartic acid and glutamic acid

c) t-butoxycarbonyl (Boc) for lysine, histidine and tryptophan

d) 2,2,5,7,8-pentamethylchroman-6-sulfonyl (Pmc) for arginine and

e) trityl (Trt) for cysteine.

The protected amino acids are activated *in situ* by addition of the activator, DIC and the additive, 1-hydroxy-benzotriazole (HOBt).

Synthesis of the peptides is accomplished by repetitive cycles of Fmoc-deprotection, washing and AA couplings, adding one amino acid per residue per cycle. After completing the synthesis of the desired peptides, the final F-moc protecting group is removed and the terminal group can be capped by acetylation. Removal of side-chain protective groups is effected by trifluoroacetic acid containing one or more scavengers.

2.3.2 Selection of synthesis regions and generation of the synthesis schedule

The sequence for synthesis was obtained from GenBank (Accession No. P02649) and an internet protscale (http://www.expasy.ch/cgi-bin/niceprot.pl?) analysis, based on the method of Hopp and Woods (1981, 1983). The SwissProt program requires the use of single letter AA codes of the protein sequence for analysis (Appendix 5).

13-mers overlapping by 6 amino acid peptides of Apo-E were synthesized

MKVLWAALLV[10]	TFLAGCQAKV[20]	EQAVETEPEP[30]	ELRQQTEWQS[40]
GQRWELALGR[50]	FWDYLRWVQT[60]	LSEQVQEELL[70]	SSQVTQELRA[80]
LMDETMKELK[90]	AYKSELEEQL[100]	TPVAEETRAR[110]	LSKELQAAQA[120]
RLGADMEDVC[130]	GRLVQYRGEV[140]	QAMLGQSTEE[150]	LRVRLASHLR[160]
KLRKRLLRDA[170]	DDLQKRLAVY[180]	QACARECAER[190]	CLSAIRERLC[200]
PLVEQCRVRA[210]	ATVCSLACQP[220]	LQERAQAWCE[230]	RLRARMEEMC[240]
SRTRDRLDEV[250]	EQVAEVRAK[260]	LEEQAQQIRL[270]	QAEAFQARLK[280]
SWFEPLVEDM[290]	QRQWAGLVEK[300]	VQAAVGTSAA[310]	PVPSDNH[317]

Schedule generation was aided by the Multipin Peptide Synthesis software package developed by Chiron, requiring input information of the whole AA sequence of epitope, concentration of AA's used for synthesis (60 mM), length of the peptides to be synthesized (317 AA), the AA's overlap (6), and the number of duplicates of the sequence in a single pin. Once all the parameters were entered and checked, a total of 44 peptides were generated. The synthesis schedule is shown in Appendix 6.

The relevant information regarding requirements of a day's synthesis, which includes appropriate amounts of Hydroxybenzotriazole (HOBt), diisopropylcarbodiimide (DIC) and all the amino acids needed. The required chemicals and AA were weighed as recommended by the schedule and were stored in clean, dry and airtight bottles. This synthesis schedule also provides information on the specific position of wells in the reaction tray in which to dispense AA.

2.3.3 Pre-synthesis preparations

a) The peptide synthesis kit (Figure 2.2) from Chiron Technologies consisted of polyethylene pins, blocks, reaction trays, sealable containers, control (positive and negative) pins, control antibodies (all of which were stored at 4 °C) and the peptide synthesis software.

b) The success of the synthesis depended heavily on clean, non-contaminated pins properly stored in sealed containers at all times and surgical gloves were worn at all times when handling the pins.

c) The pins were fitted tightly onto "blocks" to make up a 7 X 12 configuration corresponding to that of a standard microtiter plate (Figure 2.3). To ensure easy identification of blocks and proper orientation of plates and pins, adhesive labels were used.

d) The numbering system of the wells used in the synthesis program did not conform to the numbering and lettering of a standard ELISA plate. The system used is illustrated in Appendix 7.

Figure 2.2: The Multipin Peptide Synthesis kit from Chiron Mimitope Peptide System

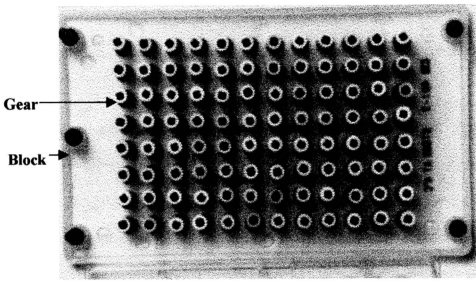

Figure 2.3: Pins on the block with gears pointing upwards

2.3.4 Weighing amino acids and activating chemicals

Based on the synthesis schedule, F-moc (9- fluorenylmethyloxycarbonyl) protected amino acids (Chiron Technologies, Australia) and HOBt (1-hydroxybenzotriazole) (Chiron Technologies, Australia) were weighed into thoroughly cleaned MacCartney bottles and then stored at 4 °C. During the weighing process, care was taken to avoid cross-contamination of amino acids by rinsing the spatula in ethanol between weighing.

2.3.5 N, N - Dimethylformamide (DMF) filtration

N, N- Dimethylformamide (DMF) is the preferred solvent for use in F -moc chemistry. The DMF used in the synthesis must be pure and free from amines. This is because in the presence of light at room temperature, DMF slowly decomposes liberating dimethylamine which is most efficient at cleaving the highly labile F-moc protecting group. To avoid this happening at the wrong time during synthesis, it is necessary to use freshly purified DMF. DMF is purified using a freshly packed column containing aluminium oxide (2/3 acidic, pH 4.5; 1/3 basic, pH 9.5, whereby the basic alumina is placed at the top of the column).

2.3.6 Fmoc-Deprotection of Pins

Deprotection is the removal of the N-terminal protecting group after coupling, in preparation for the addition of the next amino acid.

The pins which was supplied were ready for use, and fit onto a "block", configured to fit onto a 96-well microtiter plate. The pins which were used were manufactured Fmoc-protected, making them very base-labile. Piperidine, a secondary base, is used to remove the Fmoc group according to the procedure described below:

Pins were placed with the gears fully immersed into a bath containing 20% (v/v) piperidine (Sigma Chemical Co., USA) in DMF for 20 minutes at room temperature. Pins were removed, excess liquid shaken off and then immersed again in fresh DMF bath for 2 minutes at room temperature, with agitation. The solution should be sufficient to immerse gears fully.

Again the pins were removed and excess liquid shaken off. Pins were then immersed completely in methanol for 2 minutes. This washing process was repeated twice with fresh methanol each time. Pins were then removed, air-dried in a fume hood for at least 30 minutes at room temperature.

These N-Fmoc deprotection steps were carried out before starting AA coupling and were repeated daily before each coupling step was carried out. While the pins were drying, amino acid solutions required for the coupling steps were prepared and dispensed as needed.

2.3.7 Coupling the N-α-Fmoc-protected amino acids

Prior to the coupling process, all the AA's have to be activated. AA for a coupling reaction were taken out of storage and allowed to thaw at room temperature. Working solutions were: i.e. AA at 60mM; diisopropylcarbodiimide (DIC) (Millipore, USA) at 66mM and N- hydroxybenzotriazole (HOBt) (Chiron, Australia) at 120mM.

The procedure starts with the preparation of HOBt and DIC solutions using filtered N, N- dimethylformamide (DMF) (Sigma, St. Louis, USA). The amount of HOBt and DIC needed were measured and dissolved in purified DMF according to the amount in the synthesis schedule. Both reagents were fully dissolved in the DMF before being used to prepare the activated amino acid solutions. The HOBt - DMF solution was then added into each pre-weighed AA for the day. AA was thoroughly and homogeneously dissolved by vortexing. The addition of solution of DIC - DMF would trigger the activation of the AA.

The AA's were dispensed following a recommended order of specific sequence, as shown in Appendix 8. These activated AA was then dispensed into respective wells of the reaction tray as indicated by the synthesis schedule. The pin block was then submerged into the reaction wells, at correct orientation. The tray and block were kept at 4 °C (RT) overnight in a sealable plastic container.

2.3.8 Washing of synthesized pins

After the overnight incubation to couple the first AA, the block of pins was taken off the reaction tray to stop the coupling reaction and processed. Washing was carried out according to the following procedure:

The block was placed in a methanol bath in which the pins were immersed to half their height (completely covering all the gears) and agitated for 5 minutes. Excess methanol was flicked off and the pins allowed to air-drying for 2 minutes.

The block was then placed in a DMF bath in which the pins were immersed to half their height and washed with agitation for 5 minutes.

The next cycle can commence immediately after these washing steps. The deprotection, washing, coupling and washing steps were repeated until all the AA's were coupled.

2.3.9 Acetylation of terminal amino groups

On completion of the 13th day of synthesis, the N-terminus of each peptide was routinely acetylated in order to remove the charge associated with a free terminal amino group. Acetylation was carried out after the Fmoc - deprotection and the washing procedure of the last coupled AA. Thus, after the final coupling, the pins were washed and Fmoc - deprotected, and then subjected to acetylation of amino terminal groups. The block was placed into a reaction tray containing 150 µl/well of the following acetylation mixture:

DMF : Acetic anhydride : N - ethyldiisopropylamine (193: 6 : 1 v: v: v)

The tray containing the block was placed in a sealable polypropylene bag or container and the reaction allowed to proceed for 90 minutes at room temperature. At the end of the incubation period, the block was removed and washed in methanol bath for 15 minutes, followed by air-drying for at least 15 minutes. The peptides bound to the pins now ready for side-chain deprotection.

2.3.10 Side - chain deprotection

After the acetylation step, the air-dried pins were subjected to side-chain deprotection. All the protecting groups used to protect side - chain functionalities during synthesis had to be removed from the synthesized peptides prior to ELISA testing. Side - chain deprotection was accomplished by treating the pins with the following cleavage mixture:

Trifluoroacetic acid : ethaneditiol : anisole

38 : 1 : 1 (v: v: v)

The pins were treated in a polypropylene bath for 2 1/2 hours at room temperature. After removing the blocks from the deprotection bath, the pins were then allowed to air-dry for 10 minutes, and then sonicated in 0.1 % HCl methanol / distilled water (1 :1) for 15 minutes. Following this, the pins were allowed to air dry. The block was stored in a sealable container with a desiccant at 4 °C till needed.

2.4 ELISA against the Apolipoprotein E synthesized peptides

The ELISA protocol adapted here has been tested successfully with multipin peptide system and was designed for horseradish peroxidase-labeled anti-species antibodies with ABTS / OPD (ortho-phenyl diaamine) as the chromogenic substrate. Flowchart of ELISA testing is shown in Figure 2.4.

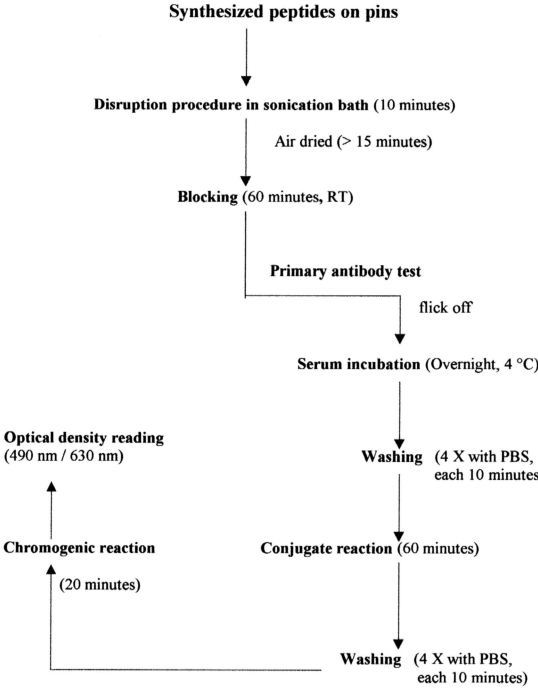

Figure 2.4: Flowchart showing Peptidylated Pin ELISA

*** Conjugate test**

The conjugate used in the Multipin ELISA protocol was an antibody, and can bind directly to the peptides. Thus, the reactivity of the peptides with the conjugate had to be checked before testing with the primary antibody was carried out. This step is more important after one cycle of screening with a primary antibody (normal human sera, atherosclerosis sera, cord blood sera) followed by the "disruption" procedure. The conjugate test performed then will indicate whether the "disruption" is complete, i.e. whether all the primary antibody bound to the peptides on the pins has been removed.

*** Pre-coating of pins (Blocking step)**

Pre-coating is essential to reduce the non-specific binding, therefore reducing the background-to-signal ratio too. To pre-coat the pins, 200 µl of precoat buffer was dispensed into each microtiter plate wells. The pins were placed in the wells (gears should be fully immersed in precoat buffer) and incubated for 60 minutes, at room temperature on a shaker platform (Belly Dancer, USA). After incubation, the pins were washed with 0.01 M PBS (pH 7.2). Washing was for 10 minutes at room temperature with agitation on the shaker platform.

*** Conjugate reaction (Secondary antibody)**

Conjugate was diluted 1:3000 / 1: 1000 with the conjugate diluent and dispensed 200 µl/well into the microtiter plate. PBS (0.01 M) washed pins were placed into the wells filled with conjugate solution and incubated at room temperature for 60 minutes with agitation on a shaker platform.

*** Substrate reaction**

Substrate solution was freshly prepared for each assay and dispensed 150 µl/well into ELISA plates. The plates were checked to ensure correct labelling and the block was oriented correctly. The pins were incubated for 20 minutes at room temperature on a shaker platform.

The pins were removed from the substrate solution to stop the enzyme action. At the end of incubation, microtiter plates was read immediately on a Dynex MRX ELISA reader, after having ensured that substrate solution were evenly mixed in the wells. Reading was done at wavelengths of 490 nm and 630 nm in a dual wavelength mode.

*** Primary antibody test**

The assay with the primary antibody is as in the standard indirect ELISA.

After the precoat blocking step, an amount of 200 µl of human serum diluted in 1: 50 (in precoat buffer + 0.1% (w/v) sodium azide) was dispensed into appropriate wells of the microtiter plates. Excess precoat buffer was flicked off from the pins, which were then placed into the wells. Incubation was done overnight at 4 °C.

PBS washes were done after every incubation with primary (first) antibody. Pins were removed from the primary antibody solutions. Subsequent washes were conduced four times in 0.01 M PBS (pH 7.2). Fresh PBS was used in every washing. Each wash was for ten minutes at room temperature with agitation on the shaker platform.

Substrate reaction was carried out as for the conjugate test.

*** Removal of bound antibodies from pin peptides (Disruption procedure)**

For reasons of reusability, it is a plus factor of the pins (having been covalently coupled to the peptides) to be able to withstand harsh treatment to remove all antibodies. The sonication bath was first rinsed thoroughly with distilled water. The pins were sonicated in a "disruption buffer" (See Appendix 9) set at between 55 - 65 °C (higher temperature would damage the peptides and lower temperatures would lead to incomplete removal of the bound antibody) for 10 minutes. After which, the block was rinsed twice in distilled water (60 °C) for 30 seconds. This process was repeated whenever there were too many bubbles from the SDS (Sodium dodecyl sulphate). The block was later placed in a water bath at 60 °C for 30 minutes. Excess water was flicked off and the pins were totally immersed in hot methanol (60 °C) for at least 15 seconds. They were then allowed to air-dry for over 15 minutes. The pins were ready for further testing.

Ideally a conjugate test should be done immediately with each block (before further testing) and if the reactivity was found to be still high after one cycle of disruption, the disruption process was repeated. When not to be used immediately, the block was stored in a sealed container with desiccant at 4 °C.

NOTE: The solutions and buffers used for conjugate test and primary antibody test are in Appendix 9.

2.5 Quality Control

To measure the quality of the synthesis, the reactivity of the positive control (PLAQ) and the negative control (GLAQ) was measured. The control peptides synthesized in each block provide a means of assessing the quality of the synthesis of each block. In addition to that, two sets of control peptide pins were supplied, one set having the positive control sequence PLAQGGGG and the other negative control sequence GLAQGGGG, differentiated from each other by their red and green colors, respectively.