Synthetic Protein Scaffolds as Tools for Detection and Modulation of Lectin-Like Oxidised Low-Density Lipoprotein Receptor 1

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

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ABSTRACT

Lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1) is a type E scavenger receptor found on the walls of endothelial cells, platelets, macrophages and smooth muscle cells among others. Technologies for sensing and inhibiting LOX-1 are limited to poorly performing antibodies and genetic knockouts and despite significant correlation with poor clinical outcomes, no LOX-1 modulation therapy has been tried in man. This thesis aims to establish whether 'Affimers'; new, small-size antibody mimetics can be used in an assay platform to detect soluble forms of LOX-1 in solution and in the modulation of its function as an oxLDL receptor. The findings show that Affimers can be used in combination with antibodies, or in complimentary binding pairs to develop a sensitive chemiluminescence based assay. Further, when used in a concentration of 500ng/ml, Affimers can inhibit the early binding of oxLDL to LOX-1, in a controlled LOX-1 cellular expression system, though their effect is nulled after prolonged incubation. Such findings along with other recently published evidence demonstrate that Affimers show great promise as diagnostic and therapeutic agents and there is enormous opportunity to develop the technology further from bench to bedside.

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ABBREVIATIONS

ADAM	A Disintegrin and metalloproteinase domain-containing protein
AGE	Advanced glycation end products
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BCA	Bicinchonic acid
BSA	Bovine serum albumin
CLEIA	Chemical Luminescence Enzyme Immunoassay
CLAAT	Chemiluminescence Affimer Affinity Test
CTLD	C-type Lectin Domain
CVD	Cardiovascular Disease
CRP	C-Reactive Protein
DAPI	4'. 6-diamidino-2-phenylindole
Di-I	1,1',di-octadecyl-3,3,3'3'-tetramethylindocarbocyanine
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
ECD	Extra-cellular domain
ECL	Enhanced chemiluminescence
ELISA	Enzyme Linked Immunoassay
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
HEK	Human Embryonic Kidney Cell
HRP	Horseradish peroxidase
ICAM-1	Intercellular adhesion molecule-1
IHD	Ischaemic Heart Disease
LB	Luria-Bertani
LDL	Low-Density Lipoprotein
LOX-1	Lectin-like, Oxidised Low Density Lipoprotein Receptor-1
MARCO	Macrophage Receptor with Collagenous Structure
MCP	Monocyte chemoattractant protein

MCSF	Monocyte colony stimulating factor
oxLDL	Oxidised Low-Density Lipoprotein
PAD	Peripheral Arterial Disease
PAEC	Porcine Aortic Endothelial Cell
PAGE	Polyacrylamide gel electrophoresis
RAGE	Receptor for advanced glycation end products
SDS	Sodium dodecyl sulfate
SMC	Smooth muscle cell
SOC	Super Optimal broth with Catabolite repression
SOFIA	Surround Optical Fibre ImmunoAssay
SR	Scavenger Receptor
TGF	Tumour growth factor
TNF	Tumour Necrosis Factor
VCAM1	Vascular cell adhesion molecule-1

CHAPTER 1: INTRODUCTION

1.1 Atherosclerosis

1.1.1 Atherosclerosis and cardiovascular disease

Atherosclerosis is a chronic, progressive, inflammatory disease of the arterial wall (Figure 1-1) which leads to stenosis and eventual occlusion of the arterial lumen (Lusis, 2000). Such arterial narrowing leads to impaired delivery of blood, oxygen and nutrients to tissues and clinically, atherosclerosis manifests itself in a variety of ways including intermittent claudication (Hiatt et al., 2015), *angina pectoris* (Vlodaver et al., 1972), renovascular hypertension (McLaughlin et al., 2000), and mesenteric angina (Sreenarasimhaiah, 2005). Further, unstable atherosclerotic plaques, discussed in more detail below, can rupture acutely, leading to near-instant vascular thrombotic occlusion or embolisation. Such events present acutely as myocardial infarction (Frangogiannis, 2015), stroke (Panel et al., 1997) and acute limb ischaemia (Creager et al., 2012). The above conditions, generally referred to as cardiovascular disease (CVD) have an enormous global impact. CVD accounted for nearly a third of all deaths globally in 2012, approximately 17.5 million people (Collaborators, 2015). The impact of strokes, heart attacks and other conditions on those who live with the conditions should not be underestimated. The loss of income and associated costs of care of stroke and heart attacks are estimated to cost approximately €110 billion per annum in the European Union alone (Wilkins E, 2017). Thus, the impact of CVD stretches beyond individuals to populations and governments. There is therefore a strong impetus to research and prevent these conditions which are brought about by atherosclerosis.

Given the enormous cost of CVD, early cardiovascular clinical research such as the Framingham cohort study, sought to identify who was at greatest risk of CVD. Indeed, this lead to the discovery of modifiable and non-modifiable 'cardiovascular risk factors' (Table 1-1) and it is now understood that the modulation of these risk factors correlates with an improvement in CVD outcomes such as death, myocardial infarction and stroke. Work from the Framingham study has been iterated upon many times and the evidence base establishing cardiovascular risk factors is growing constantly. However, despite these innovations, cardiovascular disease is still the leading cause of death internationally, leaving a great deal of scope for further research.

1.1.2 Initiation of atherosclerosis

It is widely accepted that atherosclerosis is initiated by the accumulation of lipoproteins in the sub-endothelium of the arterial wall (Williams and Tabas, 2005). The exact mechanism by which this occurs is not fully understood, but broadly, it has been attributed to injury to endothelial cells (Ross and Glomset, 1973), which is mediated by sustained exposure to oxidative stress (Deanfield et al., 2007). One theory proposes that injured endothelial cells become senescent and eventually detach (Woywodt et al., 2002), exposing the subendothelium (Figure 1.1) to circulating lipoproteins. Another theory proposes that lipoproteins are able to pass through endothelial cells via gap-junctions (Meyer et al., 1991). In truth, the mechanism influencing the translocation and retention of low-density lipoproteins in the subendothelium is likely multifactorial and more complex than either model described above (Tabas et al., 2007). Once lipoproteins enter the subendothelium, they undergo oxidation by metabolic products of the endothelium, as described in

the previous section (Witztum, 1994). These oxidised low-density lipoproteins (oxLDL), induce the secretion of monocyte chemoattractant protein (MCP-1) (Cushing et al., 1990), monocyte colony stimulating factor (M-CSF) (Rajavashisth et al., 1990) and vascular cell adhesion molecule (VCAM-1)(Khan et al., 1995) by endothelial cells, which among other signalling molecules and cytokines, act to induce monocyte adhesion to the endothelium and their infiltration into the subendothelium (Gerhardt and Ley, 2015). As they migrate, they transform into macrophages, which bind oxLDL via scavenger receptors, discussed later and engulf the molecule. Once within macrophages, oxLDL is transported to lysosomes where it metabolised to cholesterol esters, this is believed to be a protective mechanism, but the esters become sequestered within the macrophage and build up within the cell. Eventually, the metabolism of oxLDL within the macrophage becomes increasingly dysfunctional as more and more lipid builds within the cell, eventually leading them to become engorged (Bobryshev, 2006). Microscopically, such cells have a 'foamy' appearance, and are referred to as foam cells. As more LDL enters in the subendothelium, more monocytes are recruited, and more foam cells accumulate (*type I lesions)*, until eventually clusters can be appreciated macroscopically, such clusters are referred to as 'fatty streaks' (or *type II lesions*). This process begins in childhood (Berenson et al., 1998), and can almost be considered a physiological part of aging, however, over decades type II lesions can evolve to clinically relevant atherosclerosis.

Figure 1-1: Arterial Wall Layers
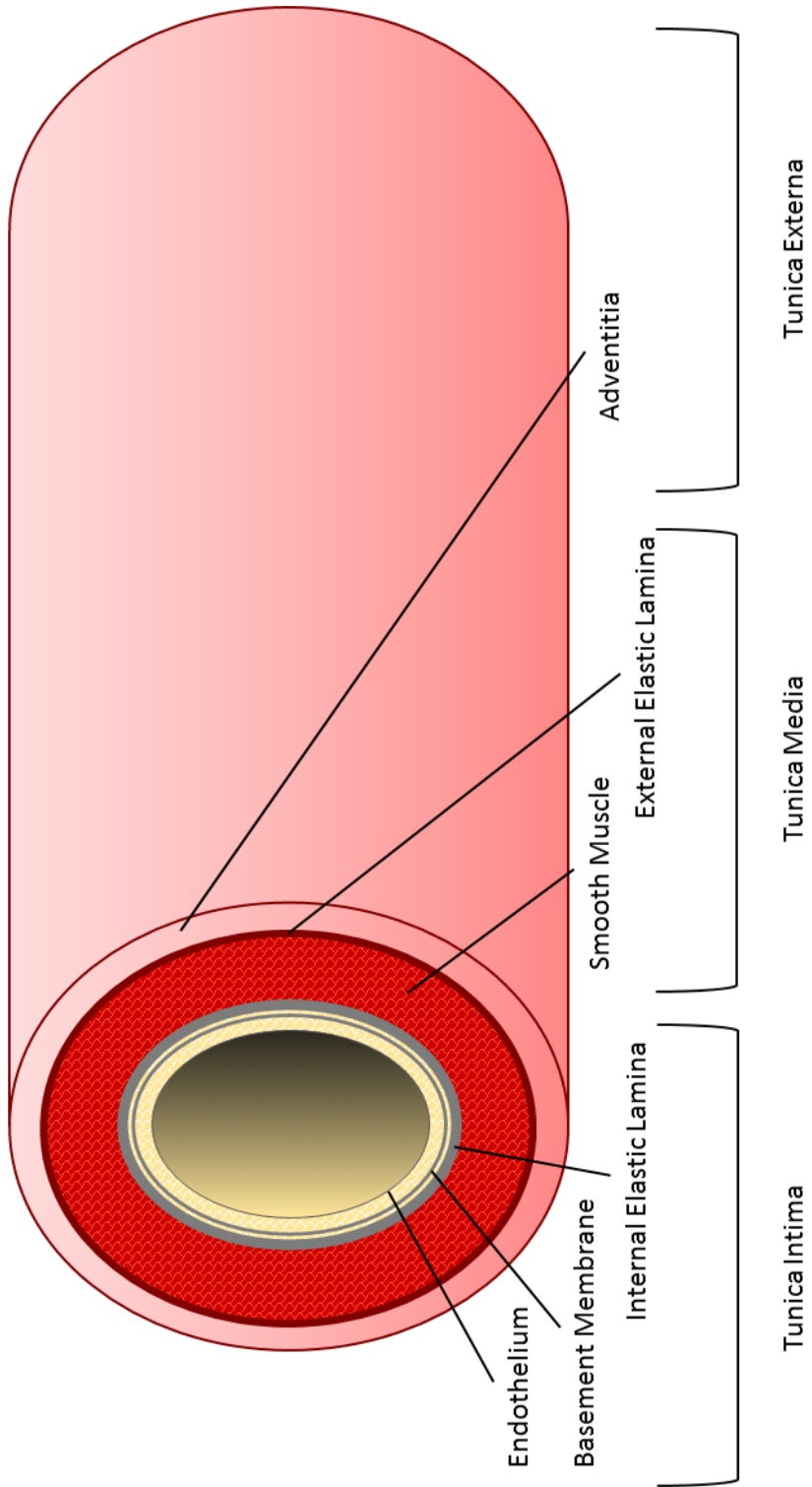
Table 1-1: Risk Factors for cardiovascular disease

Modifiable	Non-Modifiable
Hypertension (Kannel et al., 1961)	Age (Kannel et al., 1961)
Diabetes Melitus (Grundy et al., 1999)	Gender (Roger et al., 2011)
Smoking (Ockene and Miller, 1997)	Family History (Snowden et al., 1982)
High LDL / VLDL (Howard et al., 2000) (Ren et al., 2010)	Ethnicity (McKeigue et al., 1989; Wild and McKeigue, 1997)
Low HDL (Despres et al., 2000)	
Hyperhomocysteinaemia (Wilcken and Wilcken, 1976)	
Sedentary behaviour (Ford and Caspersen, 2012)	
Metabolic Syndrome (Galassi et al., 2006)	
High sensitivity CRP (Ridker, 2001)	

Abbreviations: LDL: Low-density lipoprotein, VLDL: very low-density lipoprotein, HDL: High density lipoprotein

1.1.3 Atherosclerotic plaque progression

The histological characteristics of the different developmental stages of atherosclerotic plaques have been well described by Stary (Stary, H C et al., 1992; Stary, H C et al., 1994; Stary, Herbert C., 2000). Briefly, as more monocytes are recruited, fatty streaks develop into intermediate (*type III*) lesions containing small pools of lipids and accompanying intimal thickening. Intermediate lesions can progress into pathological atheroma (*type iv lesions*). Type IV lesions contain dense lipid accumulations, referred to as lipid cores, brought about by the confluence of multiple lipid pools. This lipid core is typically lined with macrophages contributing to a progressive and often significant intimal thickening. The lipid pool also serves to displace some of the smooth muscle cells lining the intima and these cells, undergo subtle histological changes including lipid droplet inclusions. Such cells also begin to envelop the macrophage layer as do different forms of leucocytes such as an increasing population of lymphocytes and mast cells (Jonasson et al., 1986).

Type IV lesions can be unstable and are at risk of rupture, as the lesion matures into a type V lesion (*fibroatheroma*) collagen and elastin are laid down around the lipid pool, presumably as part of a reparative process, these can become mineralised with calcium. Capillaries form within this connective tissue and a wider variety of leucocytes inhabit this type of plaque.

Should type IV or V lesions fissure or ulcerate, either due to shear stress or inherent instability in the atheroma wall, the lipid core may become exposed to the vessel lumen, leading to the activation of a coagulation cascade, surface haematoma, or intravascular thrombosis. This complicated (*type iv*) lesion can lead to *in situ* or distal,

embolic, vascular occlusion(Libby et al., 2011). Occasionally, the plaque may not rupture but may continue to grow to the point that it occludes the vessel lumen without secondary thrombosis.

1.2 Lipoproteins and Scavenger Receptors

1.2.1 Low-density lipoproteins

As described, LDL has an important role in the initiation of atherosclerosis and cardiovascular disease. Lipoproteins are a group of molecules which carry triglycerides and cholesterol (needed for cell wall development) in circulating blood. Five lipoprotein groups exist: high-, low-, intermediate- and very low-density lipoproteins, as well as chylomicrons.

LDL is a large molecule, approximately 3000 kDa in size, and its primary function is to deliver lipids to tissues. Hevonoja (Hevonoja et al., 2000) has described LDL's structure as comprising of three distinct layers: the outer surface, interfacial layer and hydrophobic core. The core contains cholesterol esters and triglycerides, whereas the outer surface and interfacial layer contain a single apolipoprotein B-100 (Apo-B100) molecule, unesterified cholesterols and phospholipids. Apo-B100 serves to maintain the molecule structure as well as housing the binding site for cellular LDL-receptor (LDL-R) (Segrest et al., 2001).

The oxidation of LDL *in* vivo is not yet fully understood but is likely brought about by the action of a number of radical and non-radical oxidants including lipoxygenase (Parthasarathy et al., 1989), myeloperoxidase (Savenkova et al., 1994), superoxide generators (Steinbrecher, 1988) and peroxynitrite(Graham et al., 1993). Oxidation of LDL leads to a net negative charge to the molecule, which otherwise relies on a

positive charge over specific amino acid residues to bind to the LDL receptor (Young and McEneny, 2001). Indeed, native LDL is not associated with foam cell formation (Goldstein et al., 1979). Suggesting a role for receptors other than LDL-R in the early initiation of atherosclerosis. Such receptors are now termed scavenger receptors.

1.2.2   Scavenger Receptors

Scavenger receptors (SR) are transmembrane proteins which are linked by their ability to recognise common ligands. SRs are subdivided into ten classes (A-J) which display significant structural and sequence differences. SRs serve to bind and eliminate undesirable antigens such as bacteria, and modified 'self-molecules'; such as apoptotic cells, damaged or denatured proteins (Canton et al., 2013). There is notable overlap in the ligands which different scavenger receptors recognise, likely due to an element of immune redundancy. Of the ten classes of SRs, Class C SRs are only present in insects, and of the remaining, only six have been demonstrated to bind modified forms of LDL (Moore, Kathryn J. and Freeman, 2006), these are discussed below:

Class A consists of three separate groups of receptors (SR-As I, II, III, Macrophage Receptor with Collagenous Structure (MARCO) and Scavenger receptor with C-type Lectin (SRCL)). Class A SRs are type II membrane glycoproteins that form homotrimers. They consist of a cytoplasmic N-terminus, a single pass transmembrane region and an extracellular C-terminus (Kodama et al., 1990). Again, of the Class A SRs, only SR-AI, SR-AII and SRCL have shown binding affinity for oxLDL (Murphy et al., 2005; Ohtani et al., 2001). SR-AI and SR-AII are structurally and functionally very similar, they are found predominantly on macrophages and have a relatively low

affinity for oxLDL, whereas SRCL is predominantly expressed in endothelial cells and has a relatively high affinity for oxLDL (Levitan et al., 2010).

Class B SRs are type III membrane glycoproteins. There are four members of this class: CD36, SR-B, CD136 and LIMPII. Their structure consists of a central, looped extra-cellular domain and two transmembrane domains leading to an N and a C- terminus (Murphy et al., 2005). CD36 has the greatest volume of evidence supporting its link to atherosclerosis: whilst some studies assessing CD36 knockout mice demonstrated a lower rate of fatty streak deposition (Febbraio et al., 2000; Kuchibhotla et al., 2008), others have found that paradoxically, Apolipoprotein E / CD36 double knockout mice have higher rates of atherosclerosis (Moore, K. J. et al., 2005). Moreover, work from the same group who initially found CD36 had no effect on atherosclerosis using triple knockout SRA/CD36/ApoE knockout mice has yielded results better aligned to those of the initial studies(Manning-Tobin et al., 2009). Some reviewers have attributed this discrepancy to the existence of different strains of CD36 knockout mice (Collot-Teixeira et al., 2007).

Class E consists of a single SR – termed lectin-like oxLDL receptor 1 (LOX-1, OLR1). This type II membrane glycoprotein is explored in more depth in the next section. Initially believed to be expressed only in the endothelial cells of mammals and worms, there are two SRs in class F - Scavenger receptor expressed by endothelial cells I (SREC-I) and II (SREC-II). SREC-I is a type I transmembrane glycoprotein consisting of an N-linked extracellular domain, multiple endothelial growth factor-like domains, a transmembrane domain, a Ser/Pro-rich intracellular domain and a carboxy-linked glycine-rich domain (Adachi et al., 1997). Despite its name, recent evidence suggests

that SREC-1 is also expressed in macrophages where it is involved in the uptake of acetylated LDL (Tamura et al., 2004).

Scavenger receptor for phosphatidylserine and oxidized lipoprotein (SR-PSOX) is the only Class G SR, it was first described in 2001 (Minami et al., 2001) and is expressed in macrophages, endothelial and smooth muscle cells (Wågsäter et al., 2004) within vascular tissues. It is a 254 amino acid, type I membrane protein which includes an extracellular domain containing a chemokine-like region attached to a glycosylated mucin-like region (Shimaoka, Takeshi et al., 2000). Class H SRs include Fasciclin, EGF-like, laminin-type EGF-like and link domain-containing scavenger receptor-1 and -2 (FEEL-1, FEEL-2). They are the largest SRs, containing more than 2500 amino acids. Evidence suggests they have a role in binding of advanced glycation end products (AGEs) as well as acetylated LDL (Tamura et al., 2003). Lastly, Receptor for advanced glycation end products (RAGE), has recently been classified as a class J SR (PrabhuDas et al., 2014). There is evidence that oxLDL may be a ligand for RAGE due to the signalling effects brought about by its exposure (Sun et al., 2009), though this has not been thoroughly tested to date.

1.3   Lectin-like Low Density Lipoprotein Receptor-1 (LOX-1)

1.3.1   Structure and physiological function of LOX-1

The structure of the lectin like oxidised low-density lipoprotein receptor (LOX-1) (Figure 1-2) was first elucidated by Sawamura and colleagues, more than 20 years ago (Sawamura et al., 1997). LOX-1 has been identified on macrophages, platelets, endothelial and vascular smooth muscle cells (Chen, M. et al., 2001; Yoshida et al., 1998). It is a single pass, type II membrane protein belonging to the C-type lectin

family. It consists of an N-terminal cytoplasmic domain, a transmembrane domain, a short neck domain and a lectin-like domain, it is made up of 273 amino acids and has a molecular weight of 48kDa. In contrast to other scavenger receptors, LOX-1 has a structure and sequence similar to a class of immune receptors found on natural killer (NK) cells. In support of this association, the LOX-1 gene locus in humans (*OLR1*) is located within the Natural Killer receptor gene cluster on the short arm of human chromosome 12 (12p13.1-p12.3) (Yamanaka, S. et al., 1998; Aoyama et al., 1999). The *OLR1* gene consists of 6 exons and 5 introns and includes over 7000 base pairs (Aoyama et al., 1999). The first three exons code the cytoplasmic and neck domains of the protein, while the second three account for the C-type Lectin Domain (CTLD). Numerous polymorphisms of the *OLR1* gene have been reported, though the overall significance of these is uncertain (Sentinelli et al., 2006; Palmieri et al., 2013; Biocca et al., 2009). A splice variant of LOX-1 (LOXIN), lacks part of the CTLD structure because of a deletion of exon 5. This variant is believed to be protective against atherosclerosis (Mango et al., 2011).

Like other scavenger receptors, LOX-1's chief physiological function is believed to lie within the domain of innate immunity. Parlato and colleagues recently demonstrated that LOX-1 is responsible for antigen presentation in dendritic cells (Parlato et al., 2010). This mirrors earlier research demonstrating the role of LOX-1 in the destruction of apoptotic cells (Oka et al., 1998) and heat shock proteins (Delneste et al., 2002) by means of cross priming of the major histocompatibility complex class 1 pathway. More recently, it has been shown that LOX-1 also binds bacteria, advanced glycation end products (Jono et al., 2002; Shimaoka, T. et al., 2001).

1.3.2   LOX-1-oxLDL binding and signal transduction

Ohki and colleagues (Ohki et al., 2005) have proposed that LOX-1 binds to an α-helix on the surface of oxLDL, which is a much larger molecule. Further, LOX-1's arrangement as membrane-based homodimers is vital to increasing its binding affinity (Ohki et al., 2011).

As well as binding oxLDL, the expression of LOX-1 is also upregulated by it. Other molecules that stimulate LOX-1 expression are inflammatory cytokines and reactive oxygen species (Wang et al., 2011; Minami et al., 2000). Once activated, LOX-1 is associated with an increase in vascular smooth muscle tone through its activation of arginase-II(Ryoo et al., 2011), which in turn leads to a decrease in nitric oxide levels, a physiological vasodilator. LOX-1 is implicated in smooth muscle and endothelial cell apoptosis through a number of signalling pathways, including the downregulation of inhibitory apoptotic protein-1, upregulation of caspases and Bax signalling (Chen, J. et al., 2004; Kataoka et al., 2001). Conversely, LOX-1 stimulates smooth muscle proliferation and the adhesion and aggregation of platelets and monocytes in atherosclerosis (discussed in more detail below), through its activation of protein kinase C (Eto et al., 2006) and in turn, mitogen activated protein kinase and nuclear factor kB (Figure 1-3).

Figure 1-2: Lectin-like Low Density Lipoprotein Receptor-1

A)
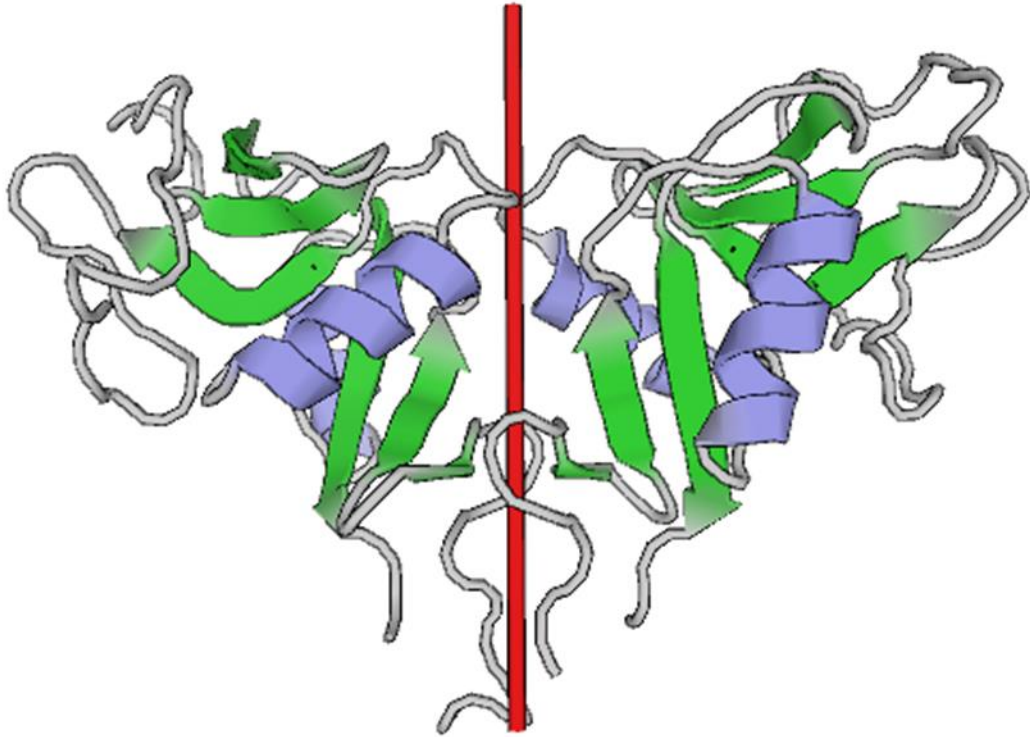
B)
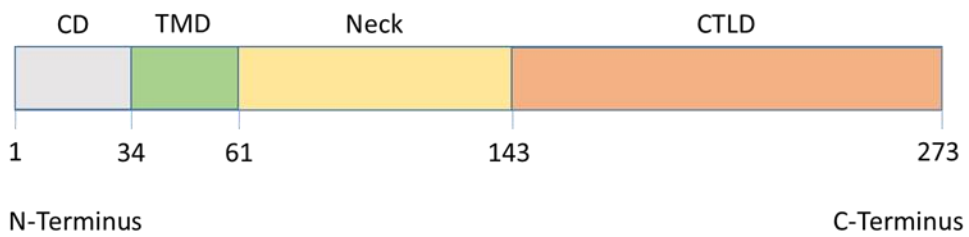
Legend: A) Crystal Structure of LOX-1 disulphide-linked dimer (Ohki et al., 2005) from *Research Collaborative for Structural Bioinformatics Protein Database* (PDBID 1YXK). B) Sub structure by residue number

Figure 1-3: LOX-1 Signalling (adapted from De Siqueira et al, 2015)
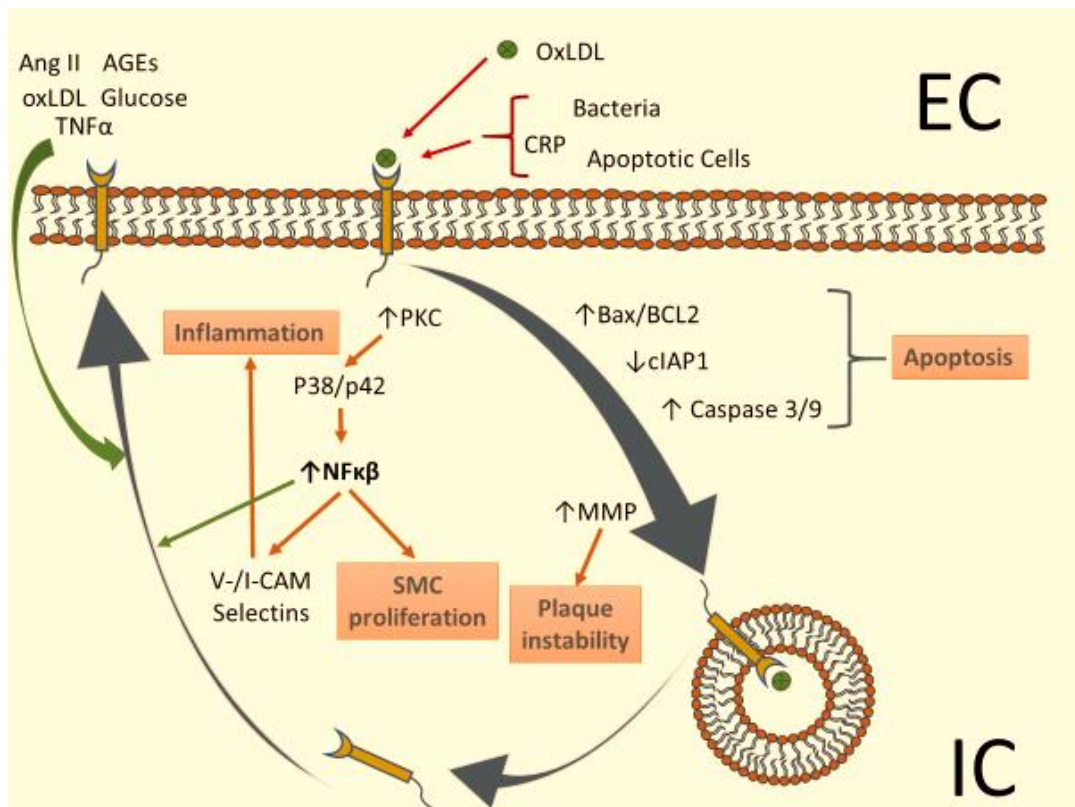
LOX-1 expression is stimulated by Angiotensin-II, oxLDL and other factors as illustrated. LOX-1 binds oxidised low density lipoproteins, CRP, apoptotic cells and bacteria. Signalling from LOX-1 stimulates a number of pathways, leading to inflammation, smooth muscle cell proliferation, cellular apoptosis and atherosclerotic plaque instability

Abbreviations: EC – Extracellular space, IC – Intracellular Space, Ang II – Angiotensin II, AGE – Andanced Glycation End Products, TNF – Tumour Necrosis Factor, CRP- C-Reactive Protein, PKC-Protein Kinase C, NFkB - nuclear factor kappa-light-chain-enhancer of activated B cells, VCAM - Vascular cell adhesion protein, ICAM - Intercellular Adhesion Molecule, SMC – Smooth Muscle Cell, MMP – Matrix Metalloprotease

1.3.3   LOX-1 and atherosclerosis

LOX-1 contributes to atherosclerosis and ischaemic events by a number of mechanisms:

Firstly, LOX-1 is responsible for an increase in endothelial mediated vascular tone through down-regulation of nitric oxide release (Ryoo et al., 2011). Additionally, oxLDL, bound by LOX-1 has been shown to bring about endothelial cell apoptosis by inhibition of antiapoptotic caspases (Chen, J. et al., 2004). Such processes bring about endothelial dysfunction and senescence, thus contributing to the start of atherosclerotic plaques. There is evidence that inhibition of LOX-1 with anti-LOX-1 antibodies leads to decreased monocyte migration to the subendothelium, implying a further association between LOX-1 expression and monocyte vascular invasion (Honjo et al., 2003).

LOX-1 facilitates the formation of foam cells by promoting the uptake of ox-LDL into macrophages (Yoshida et al., 1998). It has an additional role in mediating smooth muscle cell (SMC) proliferation and intimal hyperplasia (Hinagata et al., 2006; Eto et al., 2006). Though not shown to be brought about by LOX-1, oxLDL exposure is linked to apoptosis in vascular SMCs (Hsieh et al., 2001). Macroscopically, this leads to atheromatous plaque destabilisation and arterial thromboembolic events (van der Wal and Becker, 1999). The high levels of LOX-1 expression in activated platelets within atherosclerotic thrombus has been correlated with a relationship between platelet activation and LOX-1 expression. Since platelets are ligands to LOX-1 at other sites, it is likely that LOX-1 plays a role in the aggregation of platelets at atherosclerotic plaques (Kakutani et al., 2000).

1.3.4   Clinical Impact of LOX-1:

LOX-1 expression is upregulated in the presence of many disease states including hyperglycaemia (Li, L. et al., 2003), renal failure (Ueno et al., 2003), hypercholesterolaemia (Chen, H. et al., 2000), hypertension (Nagase et al., 2000), as well as in the presence of proinflammatory cytokines such as TNF-alpha (Kume et al., 1998), growth factors (Minami et al., 2000), and atherogenic products such as oxLDL itself (Li and Mehta, 2000). LOX-1 expression levels are elevated in atherosclerotic plaques compared to normal endothelial tissue (Kataoka et al., 1999).

Mango et al (Mango et al., 2003) have identified 7 single nucleotide polymorphisms in the OLR1 gene. One case/control study found a statistically significant correlation between one polymorphism (rs11053646), stroke risk and recovery (Zhang, J. et al., 2013). Tatsuguchi et *al* (Tatsuguchi et al., 2003) have shown that the G501C gene polymorphism is twice as common in populations with ischaemic heart disease as in those without. Lastly, the WISE study (Chen, Q. et al., 2003) identified a correlation between polymorphisms in the *OLR1* gene locus and degree of coronary artery stenosis.

A soluble form of human LOX-1 (sLOX-1) was identified in 2000 (Murase et al., 2000). Although the exact mechanism for the cleavage of LOX-1 remains elusive, recent evidence suggests that it likely takes place at the neck domain by the ADAM-10 metalloprotease (Mitsuoka et al., 2009). The significance of this molecule is discussed later.

1.3.4.1 Mammalian Studies and LOX-1

To date, four mammalian species have had their LOX-1 protein sequence elucidated (Fig 1-4). Following the sequencing of human LOX-1 (Sawamura et al., 1997), Murine LOX-1 was first sequenced in 1998 (Hoshikawa et al., 1998), this was followed by rat LOX-1(Nagase et al., 2000) and bovine LOX-1(Kume et al., 1998). The amino acid sequence of rabbit LOX-1 was published in 2000 (Chen, M. et al., 2000). Mouse and rat LOX-1 have key differences from human LOX-1: Mouse and rat LOX-1 display a structure with three repeating helical neck domain units (in contrast to a single helical unit in human LOX-1) and either a 57% (mouse) or 67% (rat) congruence with human c-type lectin-like domain sequence.

Mehta, Sawamura and colleagues were the first to study transgenic LOX-1 knockout mice (Mehta, J. L. et al., 2007). They observed that such mice demonstrated a lower rate of fatty streak deposition than wild-type. By the same vein, other studies have demonstrated that up regulation of LOX-1 has led to increased atherosclerosis in mice (White et al., 2011; Akhmedov et al., 2014). Hu found that atherosclerotic plaques in LOX1 knockout mice were richer in collagen than those in wild-type mice (Hu et al., 2008). This collagen-rich (vs cholesterol-rich) plaque is more stable and less prone to rupture and symptomatic ischaemic events, supporting previously published data on the effects of LOX-1 has on plaque instability (Hsieh et al., 2001).

Figure 1-4: Amino Acid (AA) Sequence of LOX-1 across species
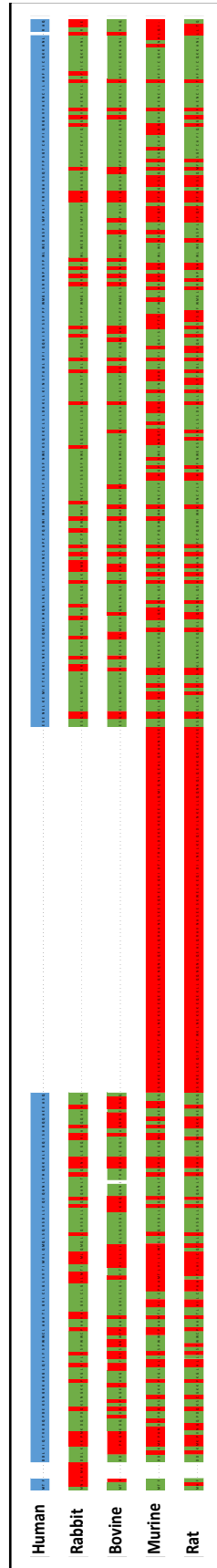
Legend: Blue: Human LOX-1 AA Sequence, Green: Matching animal AA, Red Different animal AA. Adapted from De Siqueira et al 2015

Whilst transgenic models have heralded discoveries regarding the role of LOX-1 in disease states, their translational application is limited. However, the use of anti-LOX1-antibodies has led to promising results: Hinagata et *al* demonstrated that by administering anti-lox-1 antibodies following angioplasty balloon injury to rat coronary vessels, they were able to reduce the level of intimal hyperplasia following injury (Hinagata et al., 2006). In a similar vein, Dominguez et *al* demonstrated that anti-LOX-1 antibodies could be used to preserve renal function (presumably through prevention of renal artery atherosclerosis) in obese diabetic rats (Dominguez et al., 2008). Xu et *al* demonstrated that anti-LOX-1 antibodies restore coronary artery relaxation in atherosclerotic prone mice (Xu, X. et al., 2007). Other investigators have demonstrated that the administration of anti-LOX-1 antibodies led to a reduction in the size of myocardial and cerebral infarcts (Li, D. et al., 2003; Akamatsu et al., 2014), early mesenteric atherosclerosis (Nakano et al., 2010), diabetic neuropathy (Vincent et al., 2009), arthritis (Nakagawa et al., 2002) and the systemic inflammatory response (Landsberger et al., 2010; De Siqueira et al., 2015).

To date, there has been a limited amount of research exploring how existing therapies modulate LOX-1. These include thiazolidinediones, (which inhibit LOX-1 expression *in vitro* independently of effects on hyperglycaemia) (Chiba et al., 2001), 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) (Kang et al., 2009), and angiotensin-II receptor blockers (Zhang, H. et al., 2012). However, such findings do little to advance therapeutic strategies in cardiovascular disease. Despite the published effects of anti-LOX-1 antibodies, none of the above findings have been taken forward into clinical trials.

1.4   Biomarkers and sensing technology

1.4.1   Biomarkers

Once myocardial or cerebral issue has infarcted from sustained ischaemia, it is unrecoverable. However, as described, it also is evident that major cardiovascular events (strokes, heart attacks etc) have a prolonged at-risk phase. There is therefore significant interest in identifying which members of any population are at increased risk of such events to direct preventative therapies. As has been established, pivotal work by the Framingham group (Kannel et al., 1961), identified key diseases and behaviours associated with future cardiovascular events and their conclusions still affect how such individuals are identified now, over 50 years since their work was published. New frontiers in this field are focused in the identification of novel molecules which when measured together, or in isolation, may correlate with atherosclerotic load or plaque instability, such molecules are termed biological markers, or biomarkers. The field of biomarkers is crowded by dozens of potential targets and has been extensively reviewed (Vasan, 2006). Key biomarkers with a significant evidence base to support their prognostic value include high-sensitivity C-reactive protein (Danesh et al., 2004), homocysteine (Homocysteine Studies, 2002), natriuretic peptides (Omland et al., 2005) and fibrinogen (Danesh et al., 2005).

Indeed, there is a proven link between serum sLOX-1 levels and the incidence of acute coronary syndrome (Hayashida et al., 2005). Further, it can be used as a prognostic marker (Kume, N. et al., 2010) for those who have suffered an ischaemic coronary event. Most recently, Inoue (Inoue et al., 2010) has suggested the LOX-1 index (Apolipoprotein B-bound LOX-1 x sLOX-1 levels) as a prognostic marker for ischaemic

heart disease and stroke. In their 11-year cohort study of over 2400 healthy participants aged 30-79, they found a significant difference in the hazard ratios for combined risk of stroke and heart attack between the highest and lowest quartile of LOX-1 index. However, there was no significant difference when looking at LOX-1 in isolation. However, the selected population, healthy Japanese males and females, may not have the same prognostic profile as western or 'high risk' individuals.

1.4.2   Immunoassays

One of the principal challenges in the discovery and quantification of biomarkers is in their 'sensing', i.e. one must have a validated, sensitive and specific test to reliably quantify the concentration of such biomarkers. Established technologies for sensing commonly involve the use of a specific immune molecule (discussed later) to bring about a change in the colour or fluorescence in a plate, after incubation or contact with a target analyte. Such technologies include Enzyme-linked Immunosorbance Assays (ELISA) (Engvall and Perlmann, 1971) Chemiluminescence Enzyme Immunoassay (CLEIA) and RadioImmunoassay (RIA) (Yalow and Berson, 1960) (Figure 1-5). The fundamental principle of these *immunoassays* is the same: an antibody is labelled with an isotope, fluorophore or enzyme which when exposed to a substrate, leads to a colorimetric or chemiluminescent reaction. Such antibodies are exposed directly or indirectly to the target (antigen) and the degree of 'change' (colour, fluorescence etc…) is quantified and correlated against concentration of antigen to which the antibody is exposed. Multiple variations on this format exist including direct, indirect and sandwich constructions Over time, this technology has been improved upon to the point where modern versions of the immunoassay (e.g.

Surround Optical Fibre Immunoassay (SOFIA)) can detect proteins at atto-gram (1x 10^{-18}g) concentration (Chang et al., 2009).

Immunoassays have notable drawbacks. Immunoassays can be relatively laborious to perform, involving multiple blocking and washing steps. Further, due to the need for prolonged incubation, they can take up to 24 hours to yield a result, which in an acute setting is less than ideal. Lastly, they require the use of antibodies, which as discussed later, involves significant cost and makes the platform susceptible to a short 'shelf-life'.

Figure 1-5: Different immunoassay formats
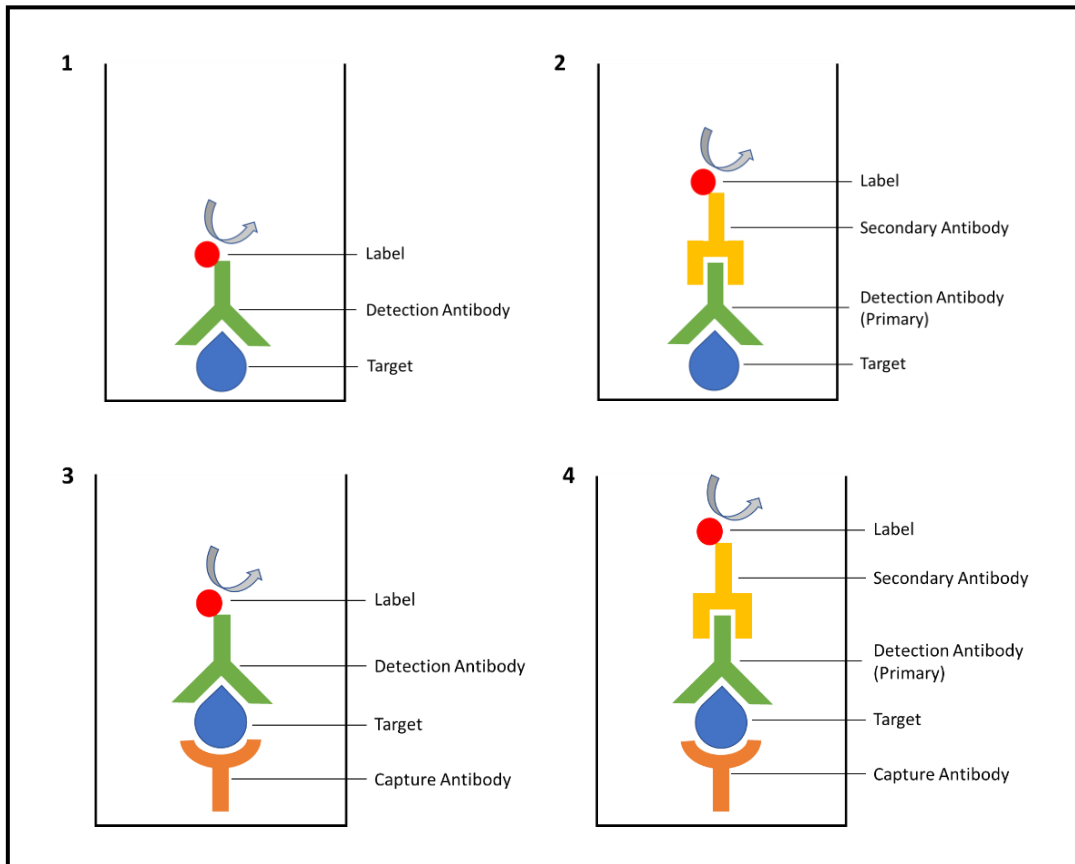
Legend: 1) Direct Immunoassay, 2) Indirect Immunoassay, 3) Sandwich Immunoassay, 4) Indirect Sandwich immunoassay

1.4.3   Biosensor principles and applications

Biosensors are an alternative, technology to immunoassays*.* Biosensors convert a biochemical reaction to a quantifiable signal. They are made in many different formats including optical biosensors, electrochemical biosensors, piezoelectric biosensors and impedance / affinity biosensors. Of these, electrochemical biosensors are the most widely used, predominantly due to their transportable and low-cost microelectronics and resistance to wave-length interference from turbid or luminescent reagents (Grieshaber et al., 2008).

Electrochemical biosensors commonly employ a recognition element (enzymes, cell receptors, antibodies) interfacing with an electrode, usually through a low impedance compound which will bind an 'inactive' epitope of the recognition element and polymerise on the surface of the electrode. The relationship between the recognition element and the electrode brings about a change in the current, charge accumulation or resistance to electrical discharge within the system (Grieshaber et al., 2008). This 'interaction' between receptor and electrode is directly affected by exposure of the receptor to the target analyte, or biomarker. Variations on the electrochemical biosensors, replaces the electrode for another form of transducer such as thermal, piezoelectrical or acoustic elements (Chambers et al., 2008).

Figure 1-6: Polymerisation Biosensor Construction
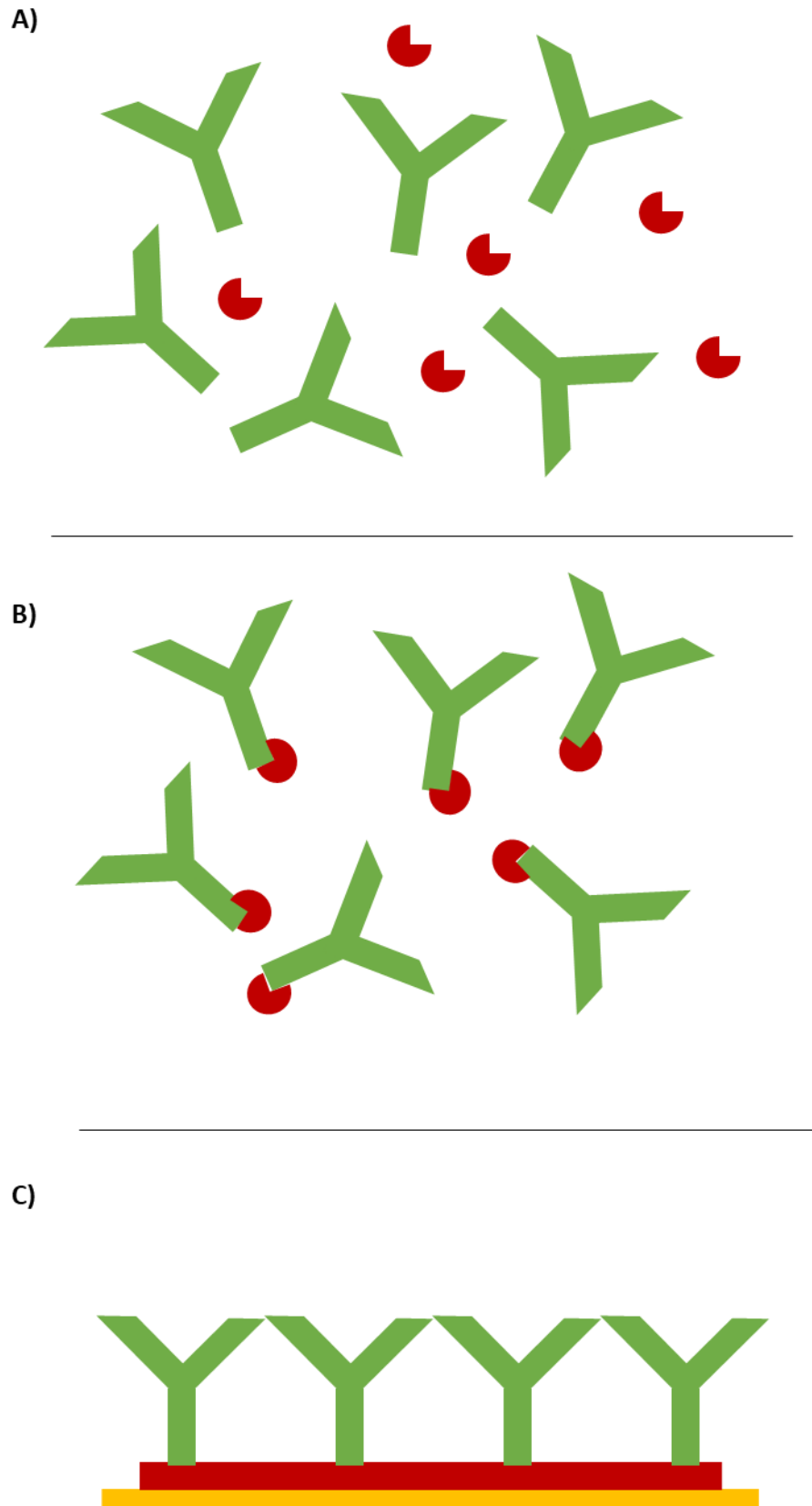
Legend: A) Receptor is incubated with polymer compound. B) Polymer compound binds 'inactive' epitope on receptor, C) compound polymerises onto electrode allowing active epitope to bind freely to target

In order to accurately measure a change in the amperometric, impedimetric and voltaic properties of the sensing (*working*) electrode, two other electrodes are often also in use during *biosensing,* these are the *counter* electrode, and the *reference* electrode. The counter electrode establishes a connection to the working solution in which testing takes place and (i.e. it is to the working electrode what the negative electrode is to the positive electrode in a simple electrolysis system). The reference electrode is kept separate from the working electrode to ascertain a stable conducting pathway with the counter electrode against which the working electrode can be measured (Figure 1-7). Due to the ubiquity of biosensors, modern sensing 'chips' are made of a pre-existing construct which include all three electrodes.

Biosensors have great potential for miniaturisation, and indeed, the oldest and most abundant biosensor, the blood glucose meter (glucometer) is widely seen in a point-of-care, miniaturised form. Developed by Clark and Lyons in the 1960s (Clark and Lyons, 1962), this biosensor consisted of an elegant modification of Clark's previously described oxygen electrode (Clark et al., 1953). This include a layer of the enzyme glucose oxidase, which coverts glucose and oxygen to gluconolactone and hydrogen peroxide. Fundamentally, exposure of the enzyme to increasing concentrations of glucose, led to increasing oxygen consumption and in turn, increasing current generation from the oxygen biosensor. Modern glucometers barely resemble Clark's original biosensor, though the principal of bringing about changes in electrical signals because of biochemical reactions has formed the foundation of an entire industry and research field. Due to the near instant results biosensors are now used in many other 'point-of-care' applications. Another notable adaptation of the Clark electrode exists in blood gas analysers (Delost, 2014) which can measure gas exchange, blood

pH, electrolyte balance, glucose and renal function simultaneously, in under two minutes. Their accuracy has also been validated by several studies (Karon et al., 2007; Jain et al., 2009). Indeed, biosensors are not only used for biomarker detection, but also in various other technological applications including food safety (Amine et al., 2006) and environmental monitoring (Rogers, K. R., 2006).

While the field of biosensors would likely not exist without Clark's pioneering work, the elegance of the glucometer is dependent on the presence of a relevant enzyme to the target analyte, which in turn could reliably and consistently promote a redox reaction. However, not all target analytes have an available redox enzyme with which to construct an equivalent sensor. This led to growing interest in *affinity biosensors*. Affinity biosensors differ from metabolic biosensors in that their construction depends on a recognition element such as an antibody or receptor protein (Rogers, Kim R., 2000). Thus, the interaction between analyte and sensing material in an affinity biosensor differs from that of a metabolic biosensor insofar as it causes a change in the electrical properties of the electrode, rather than bringing about a measurable chemical reaction.

Figure 1-7: Schematic of an affinity biosensor
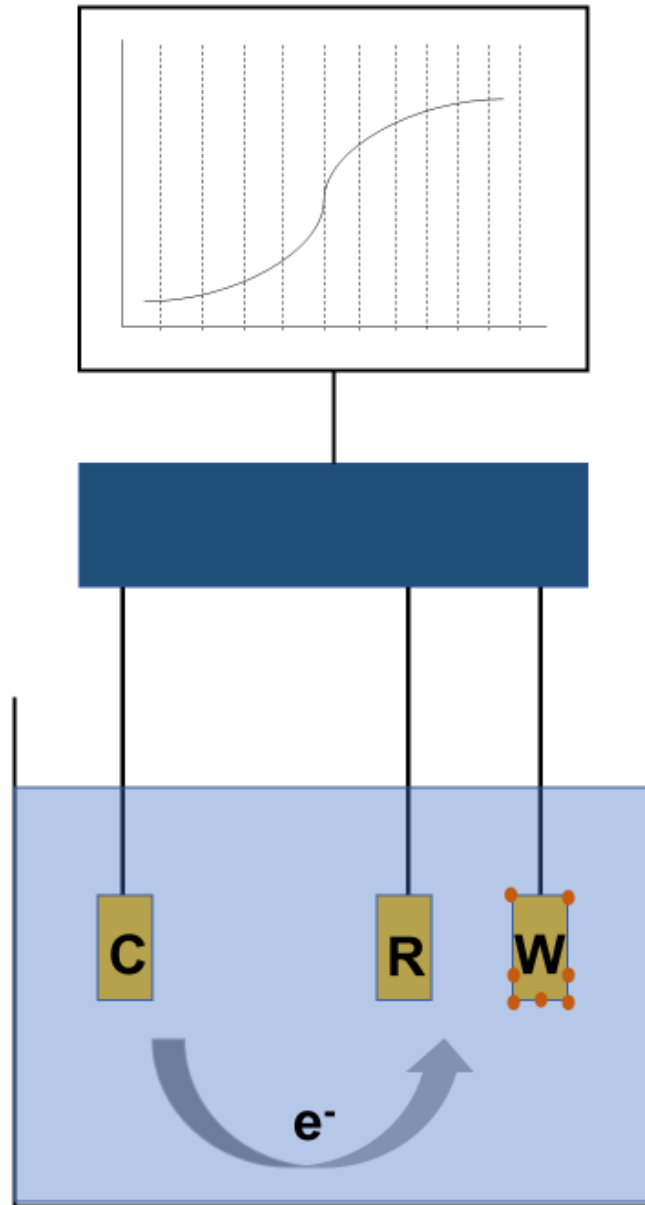
Legend: Current flows from counter (C) electrode to reference (R) and / or working (W) electrodes. The flow of current, the action potential and impedance within the system is processed by the sensing unit and displayed for the user

1.4.4   Affinity biosensor construction:

Three components must be considered when constructing a biosensing electrode: the transducer, the tethering layer and the bioreceptor itself.

The transducer is the electrical surface on which the bioreceptor is tethered to. It should be chemically inert to the reagents it is to be exposed to, though be able to form a bond to the tethering layer. It should display low impedance to electrical current and have a smooth or consistent surface to which the tethering layer can bind. Lastly, it should ideally be manufactured at low cost and consistent in properties. In practice, a transducer consists of a very fine layer of carbon, metal or polymer coated on an inert substrate, typically ceramic or plastic (Renedo et al., 2007). The coating process dictates several of the above-listed transducer properties. Among available techniques for coating, screen printing electrodes is a widely used option (Renedo et al., 2007). This process involves the coating of a suspension of gold, carbon or polymer (commonly referred to as 'ink') through a template directly onto the electrode, which is then heated. Screen printing can be a relatively low cost approach and allows for a customisation of the ink to enhance desired properties within the transducer (Yamanaka, K. et al., 2016). However, in contrast to other coating techniques, screen printing can lead to an uneven coating and a rough electrode surface (Cinti et al., 2017). By contrast, sputter coating, a form of physical vapour deposition (PVD), commonly used to prepare specimens for electron microscopy (Draggan, 1976), leads to a very smooth and even coating, though customisation of the coating material is limited.

The tethering layer binds the bioreceptor to the transducer. Like the transducer itself, the tethering layer should be relatively inert (once polymerised), have consistent conductive properties, and be applied evenly to minimise inconsistency in performance.

The experiments within this thesis employ polymerisation (Situmorang et al., 1998) exclusively. Polymerisation of the tethering layer may occur in a single- or two-step method. In a two-step polymerisation protocol, monomers of the intended polynomer are polymerised onto the transducer by cyclic voltammetry, then the transducer, coated in polymer is incubated with the bioreceptor, which will bind to free amine groups within the polymer. Alternatively, the tethering monomers, may be bound to the bioreceptor first and the whole construct may then be polymerised onto the transducer (Aydemir et al., 2016). In order to bind receptors, polymers need to have copious binding epitopes which are not involved in the polymerisation reaction. Typically, this epitope will be a primary amine (Gong et al., 2016; Xu, C.X. et al., 1997; Miscoria et al., 2006), which allows for standard cross-linking with proteins.

An alternative approach to tethering is to use self-assembly monolayer (SAM) tethering (Hammami et al., 2016). Typically, a transducer will be placed in a solution containing the tethering compound. This tethering compound will commonly contain a thiol group which will allow it to bind to a gold transducer (Vericat et al., 2010) over hours, the tethering compound will re-organise itself to form a single, even molecular layer.

Lastly, one must consider how the receptor binds to the tethering layer: in this thesis, thiol-amine coupling is employed exclusively. In this process, a coupling reagent,

containing an activated ester at one end and a maleimide group at the other, is incubated with the amine-presenting polymer and forms an amide bond via the ester group. The introduction of a thiol containing protein (typically on a cysteine residue) then allows a thiol bond to form to the other end of the coupler, at the maleimide group, conjugating the two proteins of interest (Kolodych et al., 2015). Other techniques for conjugating bioreceptors and depend very much on the particular chemical properties of the bioreceptor and tethering layer or transducer but include biotin-avidin conjugation (Chung et al., 2011), carboxyl-amine coupling (Ouellet et al., 2010) and aldehyde-amine conjugation (Marques et al., 2013)

1.4.5 Bioreceptor electrophysics

The key principle by which affinity biosensors work revolves around understanding the transfer of charge through a transducer which has a bioreceptor +/- bound target. Charge is transferred via the working electrode to an ionic solution by means of a redox reaction at the interface between the electrode and the solution. The charge is then carried through the solution and passed to the counter electrode. The key 'sensing' step relates in the interpretation of how easily charge is transferred at the working electrode. Thus, it is crucial that the ionic solution, electrodes and other components within the circuit have minimal resistance to current. A reference electrode is often used in parallel to the working electrode as a 'base-line' or negative control, against which the transfer of current at the working electrode is compared.

The data gathered by the biosensor may be potentiometric (voltage potential in a static system), amperometric (change in current as a result of a direct and static voltage potential), voltammetric (changes in current as a result of a direct application

of varying voltage potentials across the system) or impedimetric (change in conductivity (capacitance and resistance) of the system in an alternating current action potential). Potentiometric, amperometric and voltammetric biosensors rely on redox reactions at the working electrode surface to bring about changes in charge transfer and are thus are generally limited to enzyme bioreceptors. By contrast, impedimetric biosensors can employ other types of receptors (Bahadir and Sezginturk, 2016).

Impedance in a compound measurement of resistance and capacitance. To clarify, capacitance is an electrical circuit's potential for storing charge, much like a battery, whereas resistance relates to its ability to pass on charge. Resistance can be calculated by Ohm's Law:

$$R = \frac{V}{I}$$

Where: R = Resistance

V = Voltage

I = Current

However, such measurements can only be applied when voltage is constant, in direct current.

In order to measure impedance, an oscillating action potential is applied to the circuit and changes in the current in the presence of bioreceptor and target is measured. The change in current is a direct function of impedance and thus can be used to calculate it. To do so, one must account for the dynamic nature of voltage and current

within an AC circuit. Firstly, voltage and current can be calculated by the following equations:

$$V_t = V_m sin\omega t$$

$$I_t = I_m sin \omega t$$

Where: V_t = Voltage at period t (volts)

V_m = Peak voltage (amplitude) (volts)

ω = Angular Frequency (radians/second)

t = Period t (seconds)

I_t = Current at period t (amperes)

I_m = Peak current (amplitude) (amperes)

Every circuit has an element of impedance, leading to a phase shift in the current (Figure 1-8). This phase shift is accounted for by the phase angle being included in the calculation of current:

$$I_t = I_m sin(\omega t + \theta)$$

Where: θ = Phase angle (radians)

Thus, in an AC circuit, Resistance is calculated by an amalgamation of the above equations:

$$R = \frac{V}{I} = \frac{V_m sin\omega t}{I_m sin(\omega t + \theta)}$$

Figure 1-8: : Impedance effects on current.
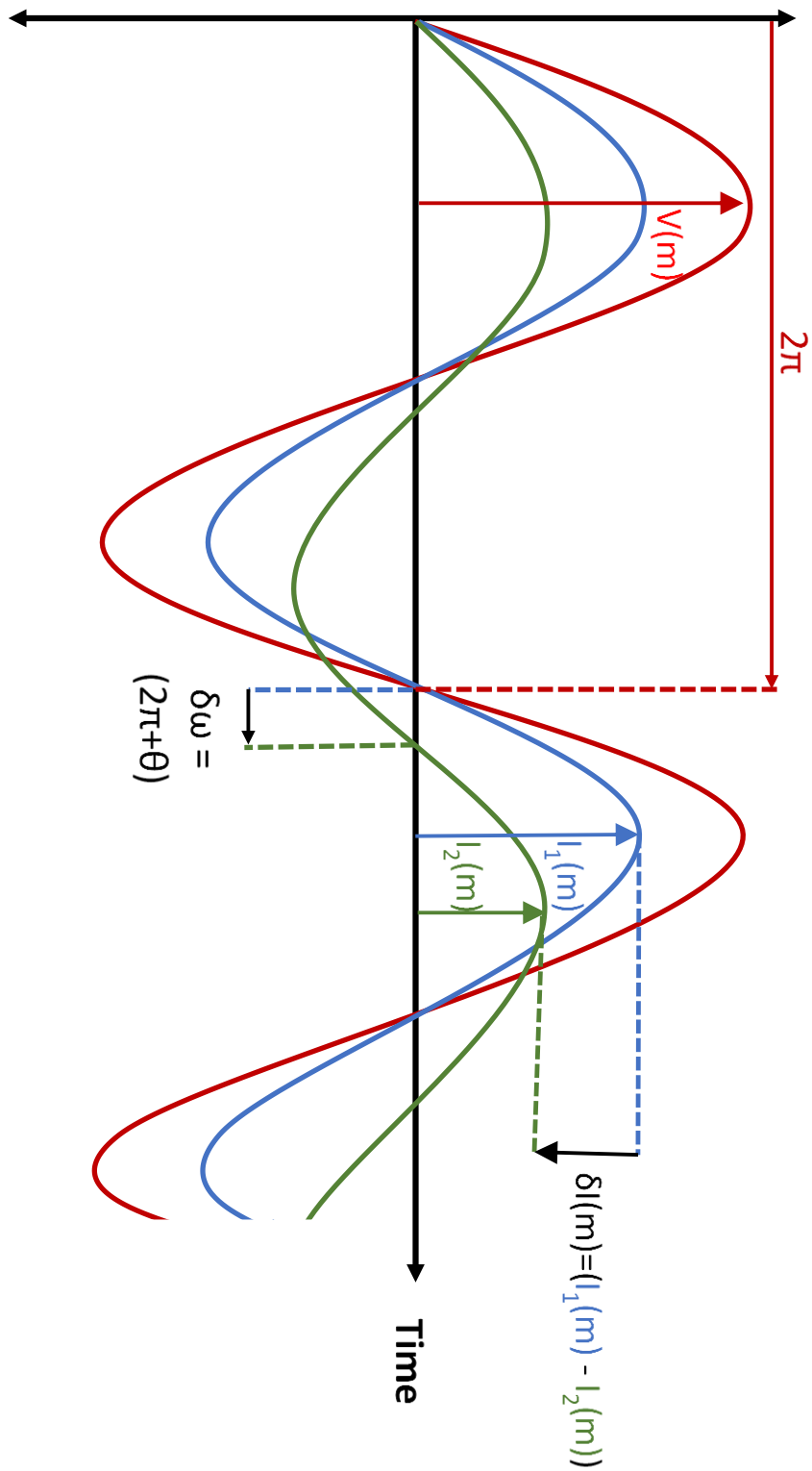
Legend: An oscillating action potential (Red) is applied to a circuit. Without impedance, current follows action potential (Blue), however impedance leads to a change in frequency and amplitude(Green). V(m): Voltage amplitude. δω: Change in angular frequency. θ: time. I(m): Current amplitude

To better understand capacitance, one must understand that it is analogous to current, a reflection of the transfer of charge over time:

$$I = \frac{q}{t}$$

Where: q = charge (coulombs)

            t = time (seconds)

However, in capacitance, there is no flow of current, as charge is stored within the capacitor. Thus, the capacitance of a system is an accumulation of charge over an action potential:

$$C = \frac{q}{V}$$

Where: C = Capacitance (farads)

A full exploration of how capacitance can be calculated in an alternating current circuit and its emergence from a system which has current within it, is complex and beyond the scope of this thesis. However, as explained, impedance has two components, resistance and capacitance. The capacitance can be calculated by the following:

$$Z = \sqrt{(z')^2 + (-z'')^2}$$

$$\therefore Z^2 = (z')^2 + (-z'')^2$$

$$\therefore (-z'')^2 = Z^2 - (z')^2$$

$$\therefore (-z'') = \sqrt{(Z)^2 - (z')^2}$$

Where: Z = Impedance

               z'= Resistance (real component of impedance)

               -z''= Capacitance (imaginary component of impedance)

1.4.6   *Interpretation of impedimetric data*

In order to fully characterise the impedance of its circuit, an impedimetric biosensor

will typically run an alternating current through its circuit at a range of frequencies.

The most common representation of impedimetric data is by means of Nyquist

plots or Bode plots. Nyquist plots chart imaginary and real impedance at increasing

frequency, Bode plots chart total impedance and phase angle shift against the log

frequency. Changes in the impedance within the circuit, brought about by target

binding at the electrode, lead to changes in the shape of the plots. Once a reliable

response in circuit dynamics can be correlated with a varying concentration of

target exposure, the biosensor can be used to interrogate unknown concentrations

of target in solution(Guan et al., 2004; Ouerghi et al., 2002).

1.5   Antibodies and antibody mimetics

1.5.1   *Monoclonal and polyclonal antibodies*

Antibodies are naturally occurring proteins which serve to detect 'non-self' (antigen)

particles and render them vulnerable to other parts of an organism's immune system.

Antibodies can be 'artificially' generated against an antigen by inoculating an

organism (e.g. mice, goats etc) with said antigen. The organism's serum is extracted

and purified to extract the antibody. An alternative method involves fusing myeloma

cells with immune cells which have been exposed to the antigen of interest. This cell

complex is then cloned, and the secreted antibodies are extracted. These two methods lead to distinct types of antibodies referred to as polyclonal and monoclonal antibodies. Polyclonal antibodies are derived from multiple cell lines and are usually a heterogenous mix of molecules which bind to different epitopes, whereas monoclonal antibodies are derived from a single cell line and they usually bind to a single epitope of the target.

1.5.2 Applications and limitations of antibodies

Monoclonal antibodies are an important tool in biological research. They are fundamental to immunoblotting (Magi and Liberatori, 2005), immunoprecipitation (Kaboord and Perr, 2008) and immunohistochemistry (Stack et al., 2014) among others. Such techniques are critical to understanding important signalling and trafficking in studied cells. Indeed, many 'bench-top' antibody applications have led to useful developments in clinical diagnostics as human blood and other fluids can be analysed by tests such as Enzyme-linked Immunoassay (ELISA) (Lequin, 2005) and immunosensors (Luppa et al., 2001). More recently, the ability of antibodies to bind to sensitively to specific cell surface proteins and disrupt their function, has led to a revolution in the approach to many diseases, including inflammatory bowel disease (Shah and Mayer, 2010), rheumatoid arthritis (Bossaller and Rothe, 2013), neoplasms (Scott et al., 2012) and more recently, hypercholesterolaemia (Sabatine et al., 2017) More recently still, there have been developments in targeted drug delivery systems, which can employ antibodies to deliver higher concentrations of disease modifying drugs to specific tissues (Firer and Gellerman, 2012).

Despite their multiple applications, antibodies have three significant limitations. Firstly, antibodies are large (150kDa), complex molecules which need extensive cell machinery to be generated. This makes them difficult and expensive to manufacture, particularly in the doses required for therapeutic benefit (Chames et al., 2009). Secondly, their large size means that penetration into target tissues can be troublesome (Beckman et al., 2007). Thirdly, commercially available antibodies for bench-top research are notoriously inconsistent and unreliable (Bradbury and Pluckthun, 2015).

Given such limitations, there is a growing body of research into molecules which can serve as antibody alternatives, such molecules are referred to as *antibody mimetics, non-antibody scaffolds* or *nanobodies.*

1.5.3   Antibody Mimetics and their Clinical Application

While an evolving field, 17 antibody mimetics have been engineered to date. Each of the binders have had varying success as antibody mimetics in basic scientific research functions. Of note, many of them have been taken forward as therapeutic agents. Kunitz domain peptides (KDP) are the basis of Ecallantide, a drug licensed for acute angioedema (Lewis et al., 2015). Depelestat, another KDP derivative was the subject of a phase II trial for respiratory distress syndrome, however, final analysis demonstrated no significant difference in outcomes compared to placebo (NCT00455767). Pegdinetanib, a monobody derivative, entered phase 2 trialling as a biological therapy against glioblastoma, though this was terminated early due to lack of efficacy (Schiff et al., 2015). BMS-986089 is an adnectin (monobody) which binds myostatin. It is currently being investigated by Bristol Myers Squibb by means of a

phase I trial in boys with Duchenne's Muscular Dystrophy (NCT02515669) Fynomers were tested as a therapeutic agent for psoriasis (COVA322), however, this trial was also terminated early due to safety concerns (NCT02243787). ABY-035, an affibody molecule also designed as a therapeutic agent for psoriasis has successfully completed a phase I, safety study (NCT02690142), and further results are awaited. A phase I trial has recently been launched for PRS-060 (Anderson et al., 2015), an inhalable anticalin engineered for the treatment of asthma. Similarly, a phase II trial of PRS-080, has completed recruitment (NCT02754167) and its results are awaited. PRS-050, an anticalin which antagonises VEGF had successful outcomes at completion of a phase I study (Mross et al., 2013), though no further studies seem to have been conducted.

Table 1-2: Antibody Mimetics

Year Published	Mimetic	Base structure	Mass (kDa)
1990	*Aptamer (Ellington and Szostak, 1990)*	RNA	5-15
2005	*Avimers (Silverman et al., 2005)*	A-Domain	4
2006	*KDP (Nixon and Wood, 2006)*	Kunitz domain	6
2006	*Affilins (Fiedler et al., 2006)*	Gamma-B-Crystallin	20
2007	*Monobodies (Koide and Koide, 2007)*	Fibronectin	10
2007	*Knottins (Gracy et al., 2008)*	Cyclotides	5
2007	*Fynomers (Grabulovski et al., 2007)*	Fyn	7
2007	*Affitins (Mouratou et al., 2007)*	Sac7D	7
2008	*Affibodies (Nygren, 2008)*	Protein A	6
2008	*Anticalins (Skerra, 2008)*	Lipocalins	20
2008	*DARPins (Stumpp et al., 2008)*	Ankyrin	10
2008	*ArmRps (Varadamsetty et al., 2012)*	Armadillo Repeat Protein	22
2012	*Atrimers (Allen et al., 2012)*	Tetranectin	60
2012	*Repebody (Lee et al., 2012)*	Lymphocyte receptors	3
2014	*Obodies (Steemson et al., 2014)*	OB-fold	12
2014	*Affimer (Tiede et al., 2014)*	Cystatin	12
2014	*Alphabodies (Desmet et al., 2014)*	Triple-helix coil	10

Pegaptanib, an anti-vascular endothelial growth factor aptamer has been successfully brought to market by Pfizer (Vinores, 2006). There has also been significant progress with Abicipar, a DARPin-based, Vascular Endothelial Growth Factor-A inhibitor: a Phase III clinical trial is currently recruiting to measure its effects on the treatment of macular degeneration (NCT02462486) (Smithwick and Stewart, 2017). A phase II trial is also due to start recruitment shortly, to assess a possible role for another DARPin, labelled MP0250, in the treatment of multiple myeloma (NCT03136653). CMX-020, a hybrid molecule based on Alphabodies bound to immunoglobulins, will shortly start recruitment for a phase II trial, assessing its effects as a therapeutic agent for pain in osteoarthritis (ACTRN12615000885594).

Not all antibody mimetics have been translated into therapeutic agents. The reasons for this variation is not immediately clear, but reasons may include a publication bias against failed attempts, immunogenic response in mammals or the lead time for such research to be published. However, despite the lack of therapeutic applications, some mimetics have been used as the recognition element in a biosensor. In particular, Aptamers (Song et al., 2008), Affibodies (Ilkhani et al., 2016) and Affimers, one of the newest non-antibody protein-binders demonstrate encouraging early results and proof-of-concept.

1.5.4   Affimers

Affimers (Adhirons) (Figure 1-9) are synthetic proteins developed at the University of Leeds by Prof. Michael McPherson and Dr Darren Tomlinson which act as antibody mimetics. Their fundamental structure is based on plant phytocystatins (Tiede et al., 2014), which inhibit cysteine proteases. However, the cystatin inhibitory sequence is

replaced with 9 randomised amino acids. This allows a large library of potential binding sequences to be generated; an M13 bacteriophage display library of over 3 billion potential Affimers has been developed. They are stable up to 101°C and can be expressed and purified from the bacterium *E. Coli*. Though an emerging technology, the use of Affimers has been proven in the inhibition of the interaction between fibrinogen and complement C3 (King et al., 2015) and, their vast phage display library has been used for identifying binding motifs in oncological drug development (Kyle et al., 2015). Indeed, a commercial licence has been issued to Avacta Life Sciences, who are actively developing the technology for diagnostics and therapeutics. Evidently, such proteins hold much promise as antibody mimetics and such potential provides viable territory in which to conduct further research.

Figure 1-9: Affimer Structure and Sequence

A
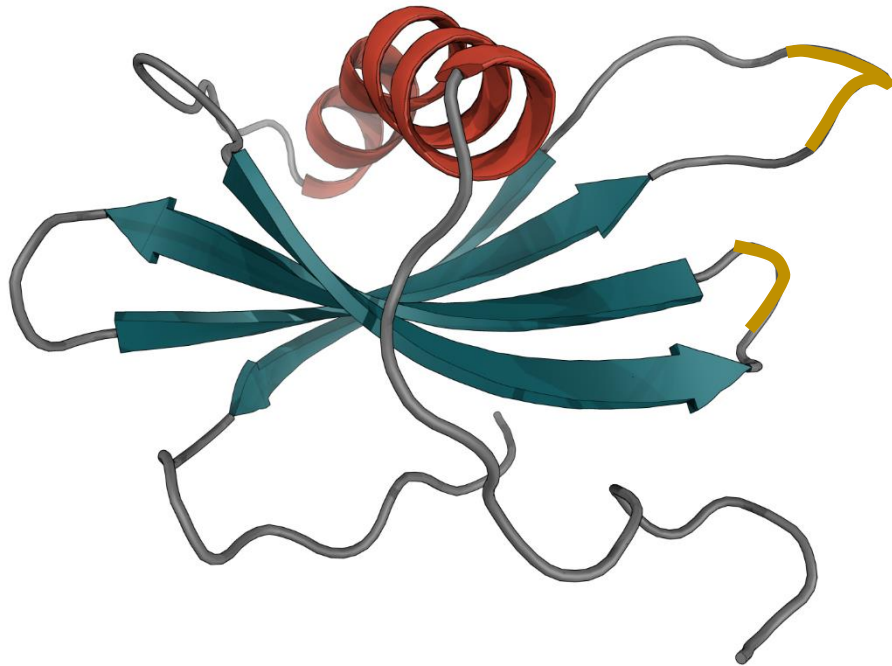
B

														*										*								*
M	A	S	A	A	T	G	V	R	A	V	P	G	N	E	N	S	L	E	I	E	E	L	A	R	F	A	V	D	E

													*										*						*
H	N	K	K	E	N	A	L	L	E	F	V	R	V	V	K	A	K	K	E	Q	–	–	–	–	–	–	–	–	–

													*										*						*
D	T	M	Y	Y	L	T	L	E	A	K	D	G	G	K	K	K	L	Y	E	A	K	V	W	V	K	–	–	–	–

–	–	–	–	–	N	F	K	E	L	Q	E	F	K	P	V	G	D	A	C	A	A	A	H	H

Legend: A) Red zone demonstrates alpha-helix, green zones beta-strands, Gold zones indicate binding sites with variable amino acid sequences. Public Domain Image, Source: Avacta Life Sciences **B)** Amino acid sequence of Affimers (dashes represent variable sequences)

1.6 Summary, Aims and Hypothesis

There is now a substantial body of work supporting the role of anti-LOX-1 antibodies in the suppression of LOX-1/oxLDL dependent signalling and in the development of histopathological changes associated with LOX-1 (De Siqueira et al., 2015). The use of antibodies as clinical therapeutic agents has expanded in recent years in rheumatology (Tanaka et al., 2014), gastroenterology (Shah and Mayer, 2010) and oncology(Scott et al., 2012), though their use in cardiovascular disease management has only recently entered major practice (Sabatine et al., 2017).

Further, anti-LOX-1 antibodies have been the basis of ELISAs upon which all studies reporting serum LOX-1 levels have been based. However, the reported normal serum levels of LOX-1 vary over more than three orders of magnitude between studies (Md Sayed et al., 2014; Tan et al., 2008), suggesting a significant inconsistency in available assays.

As discussed earlier in this chapter, antibodies have significant limitations in relation to their cost and stability. Thus, there is an imperative to investigate other antigen-binding proteins. Nearly 20 such proteins have been reported and fewer than half of these have entered trials as monoclonal antibody alternatives. This is a burgeoning field which holds much promise in both biochemical and medical technology applications.

This research project has two primary hypotheses:

1.   An Affimer-based assay can be used to differentiate between normal and elevated soluble lox-1 levels.

2.   Affimers can be used to inhibit the uptake of oxLDL by LOX-1 on the endothelial cell membrane and may have an application in modulating disease

To test the first hypothesis, the project first aimed to develop a direct, Affimer-based substitute for a conventional immunoassay and optimise this to maximise its detection limit. Should this prove successful, the Affimer-platform should be modified to a biosensor platform to test whether it could be applied more diversely.

To test the second hypothesis, LOX-1 expressing cells should be exposed to oxLDL and the effects of such exposure were to be quantified. The effect of preincubation with Affimers on oxLDL binding as well as the optimal dosing concentration were also to be calculated.

CHAPTER 2 : MATERIALS AND METHODS

2.1   Materials

2.1.1   Chemicals

All chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated; all were of analytical grade. Bicinchoninic acid assay (BCA) was from Pierce (Rockford, USA). Cell culture media and reagents were from Invitrogen (Amsterdam, Netherlands) and Promocell (Heidelberg, Germany). Enhanced chemiluminescence (ECL) reagents for immunoassay were from Thermo Fisher (Cramlington, UK) and those for immunoblotting were from Geneflow Ltd, (Staffordshire, UK)

2.1.2   Antibodies

Primary antibodies, mouse anti-FLAG, mouse anti-tubulin (Sigma Aldrich, Poole, UK) and goat polyclonal anti-LOX-1 (R+D Systems, Minneapolis, USA) were purchased commercially, unless otherwise stated. AlexaFluor-488-conjugated (Thermo Fisher, UK) and horseradish peroxidase (HRP)-conjugated (Stratech, Newmarket, UK) secondary antibodies.

Sheep anti-LOX-1 (Diagnostics Scotland, Edinburgh, UK) and rabbit anti-LOX- 1 (Eurogentec, Seraing, Belgium) were developed by the host laboratory and have been described elsewhere(Murphy et al., 2005). Working concentrations of antibodies and their use are detailed in table 2-1.

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Table 2-1: Primary and secondary antibodies listed by target, species, application and working concentration.

Antigen	Species	Source	Use	Working Concentration (μg/ml)
Primary				
α-tubulin	Mouse	Sigma Aldrich (Poole, UK), *SKU T9026*	WB	0.4
LOX-1	Sheep	S Ponnambalam (University of Leeds, UK)	WB	1
LOX-1	Sheep	Abcam (Cambridge, UK) *SKU AB111747*	WB	1
LOX-1	Rabbit	S Ponnambalam (University of Leeds, UK)	WB	1
FLAG	Mouse	Sigma Aldrich (Poole, UK) *SKU F1804*	WB	1
FLAG	Mouse	Sigma Aldrich (Poole, UK) *SKU F1804*	IF	30
Secondary				
Goat IgG	Donkey (HRP)	Stratech Scientific (Newmarket, UK) *SKU 43R-1030-FIT*	WB	0.08
Mouse IgG	Donkey (HRP)	Stratech Scientific (Newmarket, UK) *SKU 43R-1308-FIT*	WB	0.08
Rabbit IgG	Donkey (HRP)	Stratech Scientific (Newmarket, UK) *SKU 43C-CB1127-FIT*	WB	0.08
Mouse IgG	Donkey (Alexa Fluor)	ThermoFisher (Cramlington, UK) *SKU A-21203*	IF	60

Abbreviations: HRP: horseradish peroxidase IF: immunofluorescence. WB: Western blot.

2.1.3 Bacterial Strains

The XL-10 *Escherichia coli* (*E.coli*) strain Tet^{r}Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173*

endA1 supE44 thi-1 recA1 gyrA96 relA1 lac Hte [F$^-$ *proAB lacI^{q}ZΔM15* Tn*10* (Tet^r) Amy

Cam^r] was purchased from Agilent technologies (Santa Clara, CA, USA). One Shot™

BL21 Star™ (DE3) E.coli strain F$^-$*omp*T *hsdS$_B$* (r$_B^-$, m$_B^-$) *galdcmrne*131 (DE3) was

purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.1.4 Plasmids

Expression vector for extra-cellular domain LOX-1 (ECD-LOX-1)in a pET-15b (Merck

Millipore, Billerica, MA, USA) vector, previously cloned by Ravinder Vohra (University

of Leeds, UK) was kindly gifted by Dr Sreenivasan Ponnambalam, (University of Leeds,

UK). Expression vectors for Affimers A1, B1, G1, H1 and A3 were donated by Dr Darren

Tomlinson (University of Leeds, UK)

2.1.5 Mammalian Cell Lines

Human Embryonic Kidney Cells (HEK) and immortalised Porcine Aortic Endothelial

Cells(PAEC), transfected with full length wild-type mutant LOX-1 with a C-terminal

FLAG tag or empty vector (LOX-1-FLAG, EV) in the mammalian expression vector

pcDNA3 were gifted by Dr Izma Abdul-Zani, of the Ponnambalam laboratory

(University of Leeds, UK).

2.2 Experimental Methods

2.2.1 Competent bacteria preparation

BL21 Star DE3 cells were grown on Luria-Bertani (LB) agar (1% (w/v) bacto-tryptone,

0.5% (w/v) bacto-yeast extract, 1% (w/v) NaCl and 1.5% (w/v) agar, pH 7.0) overnight

at 37°C. 10 ml of LB media (1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract

and 1% (w/v) NaCl, pH 7.0) was inoculated with a single BL21 Star DE3 colony and grown for ~16 hours at 37°C on a rotary incubator at 170rpm. The bacterial culture was subsequently diluted into fresh LB (1:20) and grown to an OD_{600} of 0.6. The bacterial cell culture was placed on ice for 15 minutes and centrifuged for 5 minutes (3000 g at 4°C). Cells were resuspended in 5 ml of LB and vortexed. 2 ml of chilled Tfb I (100mM $RbCl_2$, 50mM $MnCl_2$, 30mM $C_2H_3KO_2$, 10 mM CaCl2 and 15% (v/v) glycerol, pH 5.8) was added per 5 ml of initial culture volume and cells were kept on ice for 45 min. Cells were centrifuged for 10 minutes (3000g at 4°C). Cells were resuspended in 4 ml ice-cold Tfb II (75 mM CaCl2, 10 mM RbCl2 10 mM MOPS and 15% (v/v) glycerol, pH6.5) and kept on ice for 30 min. Cells were aliquoted and stored at -70°C.

2.2.2   *DNA Transformation into competent bacterial Cells*

1.5 μl of DNA was added to 10 μl of competent E.coli XL10 gold (for bacterial plasmid replication) or BL21 *Star* DE3 (for protein expression and purification) cells and incubated on ice for 5 min. Cells were placed in a water bath at 42°C for 1 min and returned to ice for 3 min. 200 μl of SOC Media (2% w/v tryptone, 0.5% w/v yeast extract, 10mM NaCl. 2.5mM KCl, 10mM MgCl2, 10mM MgSO4, 20mM glucose) was added to cells and they were cultured on a rotary incubator (37˚C, 170rpm) for 1 hour. The cell suspension was centrifuged at 500rpm for 5 minutes and the top 100 μl of supernatant was discarded. The residual pellet and solution were vortexed. Cells were plated onto LB agar plates containing ampicillin 100 μg/ml and incubated at 37°C overnight. Plates were then stored at 4 ˚C for a maximum of two weeks.

2.2.3 DNA Purification

A single colony of E coli XL-10 gold transformed with the DNA of interest was picked from LB-agar plates and inoculated into 5 ml of LB containing ampicillin 100 µg/ml and grown at 37°C for 16 h in a shaking incubator. 1.5 ml of culture was centrifuged at 16000 g for 5 minutes to produce a bacterial cell pellet. Plasmid DNA was extracted using a miniprep kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

2.2.4 Agarose Gel Electrophoresis of DNA

2.5% (w/v) agarose gels containing 1 µg/ml ethidium bromide in 0.5x Tris-acetate (TAE) buffer (2 mM Tris, 1 mM acetic acid, 0.5 mM EDTA, pH 8) were prepared and loaded with 10 µl of DNA samples. Gels were run for 1 hour in 0.5x TAE buffer at 100 V. DNA was visualised in a G:BOX XT4 chemiluminescence imaging workstation (Syngene, Cambridge, UK).

2.2.5 Plasmid DNA Sequencing

600 ng of plasmid DNA was purified into 30 µl of deionised water using a miniprep kit, as described previously. The plasmid DNA was sequenced by the DNA Sequencing Service (Dundee University, UK) using a standard T7 primer (TAATACGACTCACTATAGGG).

2.2.6 Protein Expression

A single colony of E coli BL21 *Star* DE3, transformed with vectors of interest was picked from LB-agar ampicillin plates and inoculated into 5 ml of LB containing ampicillin 100 µg/ml and grown at 37°C for 16 h in a shaking incubator. 400ml of pre-warmed (37°C) LB containing ampicillin 100 µg/ml was inoculated with the overnight

culture and grown at 230rpm, 37°C. After 2 hours, optical density (OD) of culture was checked against uninoculated LB Broth using a NanoDrop™ (ThermoFisher) spectrophotometer at a wavelength of 600nm (OD_{600}) every 15 minutes until this reached 0.7, at this point incubation conditions were changed to 180rpm and 30°C. Once the OD_{600} was 0.8, protein expression was induced by adding Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration 0.5 mM. The culture was incubated at 30°C, 150 rpm for 6 hours. Cells were harvested by centrifuging for 15 min at 4,800*g.* Bacterial pellets were stored at -80°C until ready for purification.

2.2.7   *Preparation of Affimers for elution*

BL21 Star DE3 pellets expressing Affimers of interest were thawed in 8ml Lysis Buffer (50mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, 10% glycerol, pH 7.4), containing 0.8ml BugBuster® 10X Protein Extraction Reagent (Merck Millipore, Burlington, MA, USA) , 3.2 μl (25 U/ml) Benzonase® nuclease (Merck Millipore) and 80 μl Halt Protease Inhibitor Cocktail (100X) (Thermofisher). The suspension was incubated at room temperature for 20 minutes. Non-specific, low stability proteins were denatured by incubation in a water bath at 50°C and sonication. The suspension was centrifuged at 4000*g*. The supernatant was centrifuged further at 12000*g* using a type 45 Ti fixed angle rotor (Beckmann Coulter, Brea CA, USA). Pellets were discarded, and the supernatant containing soluble protein was used for purification of hexahistidine-tagged soluble proteins.

2.2.8   *His-tagged Affimer elution*

800μl of Ni-NTA affinity resin (Qiagen, Hilden, Germany) was washed once in 10 ml lysis buffer and twice further in wash buffer (50mM NaH_2PO_4, 500 mM NaCl, 20 mM

imidazole, 10% glycerol, pH 7.4). Ni-NTA was added to the supernatant containing soluble protein and incubated at room temperature for 2 hours. This was centrifuged at 1000g for 1 min and the supernatant discarded. The Ni-NTA was washed with wash buffer and transferred to an equilibrated 5ml Pierce Centrifuge Column (Thermo Scientific). Multiple washes with wash buffer were performed by gravity flow within the centrifuge column and the effluent's OD A280 was checked against stock wash buffer until this was less than 0.1. Elution was then performed using elution buffer (50mM NaH_2PO_4, 500 mM NaCl, 300 mM imidazole, 10% glycerol, pH 7.4). Eluates were collected 1ml at a time up to 10ml. Fractions containing an OD_{280} greater than 1.0 were pooled and dialysed into PBS over 16 hours. After dialysis, samples were centrifuged at 4000g for 5 minutes to pellet agglutinated proteins and final protein concentration was estimated using bicinchoninic acid (see below).

2.2.9   ECD-LOX-1 Expression and purification

BL21 Star DE3 cells were transformed with a pET-15b plasmid expression vector containing the DNA sequence of the extra-cellular domain of the LOX-1 protein, using procedural steps, as descried above. Protein expression and preparation followed the same procedural steps, as above, however, cells were not exposed to a 50°C water bath, as the protein of interest is more sensitive to denaturing than Affimers. Being a non-soluble protein, ECD-LOX-1 was to be found in the final cell pellet, after ultra-centifugation at 12 000g. This inclusion body pellet was resolubilised in 40 ml of solubilisation buffer (10 mM Tris, 6M guanidine hydrochloride, 100 mM NaH2PO4, pH 8.0), sonicated and mixed at 4 °C for 30 min. Finally, the solubilised inclusion body was subjected to ultra-centrifugation at 100,000g for 30 min. This supernatant was then taken forward for protein elution.

Nickel agarose column preparation and protein elution was conducted with the same procedural steps as with Affimers, however, 6M guanidine hydrochloride was added to all buffers used, due to the need to induce refolding of the protein, eluates were dialysed for 24 hours at 4°C into sequential refolding buffers over 6 days (Table 2-2). The sequence of dialysis buffers has been described by former members of the Ponnambolam laboratory (Vohra et al., 2007). After elution, the protein is unfolded by reduction of sulphide bonds, this is accompanied by suppression of ionic bonding with l-arginine. Lastly, controlled reforming of intrachain disulphide bonds and refolding takes place in a mixture of reduced and oxidised glutathione. Subsequent steps aim to gradually remove such constituents of the dialysis buffer and present the refolded recombinant LOX-1 in a workable, physiological buffer. After dialysis, samples were centrifuged at 4000g for 5 minutes to pellet agglutinated proteins and final protein concentration was estimated using bicinchoninic acid (see below).

2.2.10 BCA Assay

Total protein concentration was quantified by bicinchoninic acid (BCA) assay. 10 µl of bovine serum albumin (BSA) controls at concentrations of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml were pipetted in pairs, across the top row of a clear, 96-well polystyrene plate (Nunc, Roskilde, Denmark). Below this, 1, 3 and 5µl of the protein of interest was added. Reagents A and B of BCA protein assay (Thermo Fisher Scientific, Massachusetts, US) were mixed together in a 50:1 ratio, and 200 µl of the mixture was added into each well. The plate was incubated at 37°C for 20 min and read at 562 nm using a Varioskan Flash plate reader connected to a PC running SkanIt™ software.

Table 2-2: Composition of sequential dialysis buffers used for refolding of ECD-LOX-1.

Day	1	2	3	4	5	6
GnHCl (M)	4	3	2	1	-	-
l-Arginine (M)	0.4	0.4	0.4	0.4	0.4	-
Tris–HCl	50 mM (pH 8.5)	50 mM (pH 8.5)	50 mM (pH 8.5)	50 mM (pH 8.5)	50 mM (pH 8.5)	25 mM (pH 7.5)
NaCl (mM)	400	400	400	400	400	50
Reduced glutathione (mM)	5	5	5	5	5	-
Oxidized glutathione (mM)	0.5	0.5	0.5	0.5	0.5	-
% Glycerol (v/v)	10	10	10	10	10	-

Abbreviations: Tris: Tris(hydroxymethyl)aminomethane, APS: Ammonium persulfate, SDS: sodium dodecyl sulfate

2.2.11 SDS-PAGE

SDS-PAGE gels were prepared as per table 2-3, poured into a gel rig and allowed to set for at least 20 minutes. A 5% stacking gel (30% (w/v) acrylamide, 1 M Tris pH 6.8, 10% (w/v) SDS, deionised water, 10% (w/v) APS, TEMED) was poured over the 12-15% resolving gel. Protein samples were added to 2x SDS-PAGE sample buffer (100 mM Tris, 4% (w/v) SDS, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 4% (w/v, pH 6.8) β-mercaptoethanol) in equal volume and heated to 95°C for 5 minutes. Samples were loaded and subjected to electrophoresis in SDS running buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS) at 126V until samples reached the end of the gel.

2.2.12 Imaging of completed PAGE

Gels were trimmed to include only lanes containing protein and stained with Coomassie Brilliant Blue G-250 colloidal protein stain over four hours on a rotating shaker. Excess dye was washed with double distilled water and gels were imaged using a G:BOX XT4 Chemiluminescence imaging workstation.

Table 2-3: Composition of 12 and 15% SDS-PAGE.

Constituents	Volume	
	12%	15%
ddH$_2$O	8.9 ml	6.9 ml
30% Acryl	8 ml	10 ml
3M Tris	2.5 ml	2.5 ml
10% APS	400 µl	400 µl
10% SDS	200 µl	200 µl
TEMED	16 µl	16 µl

Abbreviations: ddH2O: Double Distilled Water, Tris:

Tris(hydroxymethyl)aminomethane, APS: Ammonium persulfate, SDS: sodium

dodecyl sulfate, TEMED: Tetramethylethylenediamine

2.2.13 Immunoblotting

Gels were transferred electrophoretically to nitrocellulose membrane (Protran 0.2 μm pore size, Schleicher & Schuell Bioscience, Dassel, Germany). Transfer occurred in transfer buffer (25 mM Tris-HCl pH 7.5, 106 mM glycine, 20% (v/v) methanol) inside a cold room (4°C) over 16 hours at 30 mA. The nitrocellulose membrane was stained with 0.1% Ponceau S in 5% (w/v) acetic acid to make bands visible and quality assured for equal loading and successful transfer. This nitrocellulose blot was then trimmed, rinsed with Tris-buffered saline with tween (TBS-T) (20 mM Tris-HCl pH 7.6, 137 mM NaCl, 0.1% (v/v) Tween-20) and blocked in 5% (w/v) skimmed milk in TBS-T for 1 hour. The nitrocellulose membrane was incubated at 4°C overnight with primary antibodies dissolved in 1% (w/v) BSA in TBS-T, containing 1 mM sodium azide. Membranes were washed in TBS-T, for 10 minutes, three times and incubated for 1 hour in secondary antibodies at room temperature. Secondary antibodies were species-specific and conjugated to horseradish peroxide (HRP). Membranes were washed again, as before and incubated with an enhanced chemiluminescence substrate detection kit (Geneflow Ltd, Staffordshire, UK) for 1 minute. Chemiluminescence was detected and analysed using a G:BOX XT4 Chemi imaging workstation. Band intensity was quantified using densitometry image analysis software (GeneSys, Syngene Cambridge, UK)

2.2.14 Affimer biotinylation

10μl of EZ-Link NHS Biotin (ThermoFisher) solution (4mg/μl dimethyl sulfoxide) was incubated with 100μl of protein in PBS over 2 hours at room temperature. Excess biotin was extracted using Zeba™ Spin desalting columns, 7K MWCO, 0.5ml (ThermoFisher). Biotinylated samples were stored at 4°C.

2.2.15 Affimer-based direct ELISA

Maxisorb 96-well plates (Nunc) were coated in triplicate with 100µl of recombinant extra-cellular domain LOX-1, diluted in coating buffer (0.39 g NaH2PO4, 1.335 g Na2HPO4, 8.474g NaCl in 1 L distilled water , pH 7.2) at 5-50000ng/ml. Plates were incubated at 4°C for 16 hours. Wells were rinsed with 200µl PBS-T (PBS with 0.1% (v/v) Tween-20) three times and blocked with 200µl 2x Casein Blocking Buffer (Sigma) over 4hours at room temperature. Wells were rinsed with 200µl PBS-T three times and incubated with 100µl biotinylated Affimer (10µg/ml in 2x Casein Blocking Buffer) at room temperature over 2 hours. Wells were rinsed with 200µl PBS-T three times and incubated with 100µl High sensitivity Streptavidin horseradish peroxidase (ThermoFisher 1:10 000 dilution in 2X Casein Blocking Buffer) over 30 minutes at room temperature. Wells were rinsed three times with 200µl PBS-T and incubated with 100µl 3,3',5,5'-tetramethylbenzidine. Colorimetry was analysed using a Varioskan Flash Multimode Reader at 370nm after 20 minutes of reaction time.

2.2.16 Affimer Sandwich ELISA

To screen for Affimers with complimentary LOX-1 binding zones. Maxisorb 96-well plates (Nunc) were coated with 100µl of cysteine-tagged Affimers diluted in coating buffer at 10µg/ml. Plates were incubated at 4°C for 16 hours. Wells were rinsed with 200µl PBS-T three times and blocked with 200µl 2x Casein blocking buffer over 4hours at room temperature. Wells were washed with 200µl PBS-T three times and incubated with 100µl recombinant ECD-LOX-1 at 500ng/ml for 2 hours. Wells were washed with 200µl PBS-T three times and incubated with 100µl biotinylated (detection) Affimer (10µg/ml in 2x Casein Blocking Buffer) at room temperature over 1 hour. The rest of the experiment was carried out as per the direct ELISA.

2.2.17 Chemiluminescence Affimer Affinity Test

The Affimer sandwich was constructed as described in the previous section but in solid-white Maxisorb plates (Nunc); in the final step, TMB was substituted with 100µl SuperSignal™ ELISA Pico Chemiluminescent Substrate (ThermoFisher). Chemiluminescence was analysed using a Varioskan Flash Multimode Reader, after 20 minutes and plates were imaged using a Syngene G-BOX.

2.2.18 Hybrid Affimer/Antibody chemiluminescence test

A chemiluminescence sandwich assay was constructed with substitution of either detection or capture Affimer by sheep anti-LOX-1 antibody (kindly donated by Mr Ravi Vohra, University of Leeds, UK). This construct was screened for capture and for detection of 500ng ECD-LOX-1 in combination with all five Affimers. Quantification was performed as before.

2.2.19 Tethering of Affimer to electrode

Affimers were conjugated to monomers by incubation with tyramine and octopamine reagents. Briefly, 1.37mg of tyramine or 1.92mg of octopamine was dissolved in 150µl of DMSO and 350µl of 100mM disodium hydrogen orthophosphate (Na_2HPO_4) (pH 7.5). 250µl of the tyramine/ octopamine solution was mixed with 2.2mg of Sulfo-SMCC dissolved in 75µl of DMSO and 175µl of 100mM (Na_2HPO_4) (pH 7.5) and incubated at room temperature for one hour. This was the tethering reagent. 200µg/ml of Affimer in 100mM HNa_2PO_4 (pH 7.5) was mixed in equal volumes with the tethering reagent over an hour at room temperature. This led to the formation of Affimer-conjugated tyramine/octopamine monomers.

Affimers/monomers were polymerised onto gold Dropsens electrodes (Dropsens, Asturias, Spain) by electrical induction. Electrodes were connected to an RA-2 AUTOLAB type III electrochemical workstation (Eco Chemie B.V., Utrecht, Netherlands), running NOVA 2 software. Two cycles of cyclic voltammetry were applied to the electrodes at 100mv per second, with a start/stop potential of 0V and a vertex potential of +1.6V. A non-specific Affimer which had been screened against green fluorescent protein (GFP) was coated onto the first working electrode (as a negative control) and a specific Affimer was coated onto the second. A characteristic curve during cyclic voltammetry, with a 'hump' forming during the first voltage cycle and a change in the shape of the subsequent cyclic wave (See Chapter 3: Results, Figure 3-6B), implied successful polymerisation.

2.2.20 Biosensor interrogation

Electrodes with tethered Affimers, as described above, were interrogated by electrochemical impedance spectroscopy. They were first incubated with PBS and then increasing concentrations of recombinant human ECD-LOX-1 (1pg/ml to 1mcg/ml in PBS) for 30 minutes at a time, before rinsing thoroughly with PBS. Electrodes were placed in a cell containing 10 mM $K_3Fe(CN_6)$, $K_4Fe(CN_6)$ and 10 mM PBS, pH 7.2. Interrogation was performed with an RA-2 AUTOLAB type III electrochemical workstation, running NOVA 2 software. Impedance analysis was performed over a frequency of 0.25 Hz to 25 kHz using a modulation voltage of 10 mV at an applied voltage of 0.0 V. Nyquist plots generated by the NOVA software were compared for differences correlating to increasing concentration of ECD-LOX-1. Experiments were repeated three times using new electrodes for experimental replicates.

2.2.21 LDL purification from blood

20ml of blood from healthy human volunteers was collected using standard phlebotomy technique (Faculty of Biological Sciences, University of Leeds ethical approval Ref: BIOSCI 15-007). To prevent clotting, trisodium citrate was immediately added to blood at a final concentration of 0.38% (w/v). Whole blood and citrate were then subjected to centrifugation at 4000rpm for 20 minutes at room temperature to pellet cells. The plasma supernatant was mixed with OptiPrep Density Gradient medium (Sigma) to give a final concentration of 12% (v/v) iodaxinol. This was layered under 1 ml of HEPES-buffered saline (HBS) (0.85% (w/v) NaCl, 10 mM HEPES pH 7.4), inside a 4.7 ml Opti-Seal centrifuge tube (Beckman Coulter, USA). Tubes were centrifuged at 100 000g for 3 hours at 16°C. Following density gradient centrifugation, LDL forms a characteristic orange/yellow band. The presence and purity of LDL in this band has been previously described and validated (Chapman et al., 1981). The LDL-containing fraction was aspirated using a 25-gauge needle, piercing the side of the tube directly to avoid contamination with other lipoprotein fractions. LDL particles were dialysed against PBS for 24 hours and its final concentration was measured by BCA, as described above. LDL was stored at 4° in 1ml fractions, mixed with 100 μM EDTA and 20 μl butylated hydroxytoluene (BHT), to prevent oxidation.

2.2.22 Oxidation of LDL

Oxidation of LDL particles was achieved by incubation with 5 μM CuSO4 at 37°C for 24 hours. Oxidation was terminated by adding 100 μM EDTA and 20 μM BHT. Final concentration was established by BCA.

2.2.23 Agarose Gel Electrophoresis of Lipid Particles

As described by Guy *et al.*, oxidation of LDL with copper sulfate, brings about greater electrophoretic mobility, in a time-dependent manner, up to approximately 20 hours, where a plateau is reached (Guy et al., 2001). Therefore, to confirm successful oxidation of lipoproteins, 4 µg of LDL and / or oxLDL particles were loaded into 0.5% (w/v) agarose gel. The gel was run in borate buffer (80 mM boric acid, 90 mM Tris-HCl pH 8.3, 3 mM EDTA) and run for 1 hour at 100 V. The gel was fixed by soaking in ethanol fixative (75% (v/v) ethanol 5% (v/v) acetic acid) for 15 minutes. Staining was achieved with a saturated solution of Sudan black in 60% (v/v) ethanol and 0.05% (w/v) NaOH for 2 hours. Lastly, the gel was destained by incubation in 50% (v/v) ethanol until clear bands could be appreciated in the gel. Successful oxidation was confirmed by the presence of separate band heights in the gel, distinguishing oxLDL from LDL.

2.2.24 Labelling of oxLDL with Fluorescent Dyes

1,1-dioctadecyl-3,3,3',3'-tetramethyllindocarbocyanine perchlorate (DiI) and 1,1-Dioctadecyl-3,3,3,3-tetramethylindodicarbocyanine (DiD) lipophilic fluorescent dyes, were dissolved in DMSO at 10mg/ml. 30µl of fluorescent dye in DMSO was mixed per milligram of lipoprotein particles and incubated in the dark for at 37°C overnight. The mixture was centrifuged at 14 000*g* for 10 minutes to pellet any excess dye and the supernatant was dialysed against PBS in for 24 hours in a sealed, dark box, stored at 4°C. Final concentration was assessed by BCA.

2.2.25 Mammalian Cell Culture

Human embryonic kidney 293 (HEK293) cell line were cultured in Dulbecco's modified eagle medium (DMEM; ThermoFisher) containing 10% (v/v) foetal calf serum (FCS; Life Technologies, Paisley, UK), 2 mM L-Glutamine, 1x non-essential amino acids, 10 U/ml penicillin and 100 μg/ml streptomycin (all from ThermoFisher). Immortalised porcine aortic endothelial cells (PAEC) were cultured in Roswell Park Memorial Institute 640 (RPMI640; ThermoFisher) containing the same additives as above. Transfected cell selection was maintained by the use of 0.5μg/ml Hygromycin B (ThermoFisher) and 0.3 μg/ml Blastacidin-S (ThermoFisher). Cell culture took place under humid, aseptic conditions at 37°C, 5% CO_2.

2.2.26 Cell Passage

Cells were passaged every 3 days. After trypsinisation with 1 ml TrypLE Express (ThermoFisher), cells were returned to incubate at 37°C for 3 minutes. 9ml of complete culture medium was used to quench the trypsin and remove the cells from the plate. Cells were split in 3:1 complete media and plated in fresh flasks.

2.2.27 Expression of LOX-1 in mammalian cells

PAECs and HEK 293-T Flp-In ™ T-Rex™ cells transfected with either FLAG-tagged, full-length, human LOX-1 DNA or an empty vector were cultured overnight in 96well plates. Media was aspirated, and cells were starved by coating with 100μl Optimem I, reduced serum media (ThermoFisher) for 2 hours. LOX-1 expression was induced by incubating with tetracycline at a final concentration of 1μg/ml.

2.2.28 Preparation of Cell Lysates

Cell-containing plates were placed on ice and all media was aspirated. Wells were washed with ice-cold PBS three times. Cells were lysed by incubating with lysis buffer (2%SDS (w/v) in PBS, 1mM phenylmethylsulfonyl fluoride) and scraping). Cell lysates were stored at -20C for a maximum of two weeks.

2.2.29 OxLDL Cell Surface Binding Assay

Induced HEK-293T and PAE-cells were placed on ice and incubated with fluorescently-labelled oxLDL particles at a concentration of 10µg/ml for 30 minutes. Cells were fixed, as described below. To assess the effectiveness of Affimers as LOX-1 receptor antagonists, experiments were repeated with pre-incubation of cells with Affimers (50pg – 500ng/ml final concentration) for 5 minutes prior to adding ox-LDL.

2.2.30 OxLDL Uptake Assay

Induced HEK-293T and PAE-cells were incubated with labelled oxLDL particles at a concentration of 10µg/ml at 37°C for fixed time periods (0,15,30,60,120 and 180 minutes) at 37°C. Plates were placed on ice and fixed, as described below. To assess the effectiveness of Affimers as LOX-1 receptor antagonists, experiments were repeated with pre-incubation of cells with Affimers (50pg – 500ng/ml final concentration) for 15 minutes prior to adding ox-LDL.

2.2.31 Affimer Toxicity Assay

To assess the toxicity of Affimers, a toxicity assay was carried out at the highest working concentration of Affimers. After trypsinisation, and splitting, 0.5 ml of cell suspension was extracted and placed in a sterile 1.5ml microfuge tube. 100 µl of cell suspension was transferred into a new microfuge tube and 400 µl 0.4% Trypan Blue

(final concentration 0.32%) was added. Trypan Blue-treated cell suspension was applied to a sterile, glass haemocytometer. Live (unstained) and dead (stained) cells were counted within four haemocytometer grids. The number of live cells were divided by the total number of cells (alive and dead) to give the cell viability. This experiment was carried out before and 24 hours after Affimer exposure and the difference analysed.

2.2.32 Cell Fixation

Media was aspirated from cells seeded on coverslips in 24-well plates. Wells were rinsed twice with 500µl PBS. Cover slips were coated with 500µl fixative (10% (v/v) formalin, Sigma-Aldrich) and incubated for 5 minutes at 37°C. Fixative was aspirated and wells were rinsed three times with 500µl PBS.

2.2.33 Immunostaining and immunofluorescence microscopy

500µl 5% (w/v) BSA in PBS was added to wells to block non-specific antibody binding, wells were then washed twice with 500µl PBS. Coverslips were removed from wells and inverted onto a 25 µl drop of primary antibody diluted in 1% (w/v) BSA in PBS. Exposure to primary antibody was conducted overnight at room temperature in a moist chamber. Coverslips were returned to fresh 24-well plates and washed 3 times with 500µl PBS. After washing, coverslips were removed from the well and inverted onto a 25µl solution containing 4µg/ml Alexa Fluor-conjugated secondary antibody and 2µg/ml 4,6-diamidino-2-phenylidole (DAPI) in 1% (w/v) BSA in PBS. Coverslips were incubated for 2 hours, in the dark, at room temperature. Coverslips were again returned to wells, washed 3 times with 500µl PBS as before and mounted onto slides

using Fluoromount G (Southern Biotech, Alabama, US). Images were acquired using an EVOS-fl inverted digital microscope.

2.2.34 Fluorescence Quantification

Di-I fluorescence was quantified using digital imaging software (ImageJ, National Institute of Health, Bethesda, USA). Captured images were uploaded into the software package and converted to 16-bit format (grey-scale). To isolate fluorescence from individual cells, image thresholds in each image were altered to create separate 16-bit particles within the image. Where significant overlap of cells was present, binary watershedding was used to separate particles. Particle thresholds were set at a minimum of 100 pixels to exclude non-cellular background fluorescence. Included particles were then used to map the area of cells in the source image and mean colour intensity was calculated per particle (cell) in the image.

2.2.35 Flow Cytometry

Flow cytometry was employed to better quantify Di-I fluorescence, using a greater number of cells. Cells for flow cytometry were subjected to the same passage, surface binding and uptake conditions as those described above. However, to allow for a larger population to be analysed, they were cultured in 6-well plates with proportional increases in all materials and solutions. After cell fixation, as described above, cells were trypsinised with 200µl of TriplE express and mechanically disrupted, then resuspended in 1ml of PBS. Cells were pelleted by centrifugation at 4000rpm for 1 minute then resuspended again in PBS. Cells were stored in 1.5ml microfuge tubes covered in aluminium foil, to prevent light degradation of labels, and stored at 4°C until cytometry could be performed (maximum three days).

Flow cytometry was performed at St James's University Hospital with the help of facility support staff. Cells were subjected to excitation with a 561nm yellow/green laser without excitation filters using an Influx Fluorescence Associated Cell Sorting system (BD Biosciences, San Jose, CA, USA) . Fluorescence was read at 570nm, in accordance with the peak fluorescence wavelength of Di-I. PAEC and HEK-293T cells expressing an empty vector, exposed to Di-I were used as negative controls, however, future flow cytometry experiments should include controls without Di-I exposure, to clarify background fluorescence of cell lines.

Cytometry data was analysed using FlowJo software (Flowjo, LLC. Ashland, OR, USA). Gating was set around forward and side scatter to exclude cell debris and applied equally across all samples for comparative experiments. Cell populations were compared by shifts in plotted fluorescence against counts and by median fluorescence.

2.2.36 Statistical Analysis

Statistical analysis of multiple groups and non-parametric variables was performed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's or Bonferroni multiple comparison test respectively. Direct comparisons between continuous variables was analysed with two-tailed Student's t-tests. Spearman's coefficient was used to analyse the correlation between LOX-1 concentration and colorimetry/chemiluminescence. Analysis was performed using Prism software (Graph-pad, La Jolla, CA, US). For all tests, α was set at 0.05. However, when calculated to be lower than 0.05, probability values were further categorised by degree (*, $p<0.05$), (**, $p<0.01$), (***, $p<0.001$).

CHAPTER 3 : AFFIMER-BASED ASSAYS FOR THE DETECTION OF LOX-1

3.1 Introduction

LOX-1 is a class E scavenger receptor found on the surface of endothelial cells, platelets and smooth muscle cells. A soluble fragment (sLOX-1) of the extra-cellular domain of LOX-1 is cleaved at cells surfaces and shed into surrounding plasma. The elevation of serum s-LOX-1 is associated with metabolic syndrome, ischaemic heart disease and stroke. However, while comparative studies demonstrate a relative increase (to healthy controls) in the serum concentration of LOX-1 in patients with the above conditions, there is much variability in the reported normal serum range. This discrepancy suggests that the assays for LOX-1 may be inaccurate, despite internal validity. Thus, there is justification for developing a new, accurate and reliable assay for soluble LOX-1.

Currently, the only commercially available sLOX-1 assays are enzyme-linked immunoassays (ELISAs). Such assays rely on anti-LOX-1 antibodies, which in turn makes them costly and sensitive to 'spoiling'. Antibodies are often derived from mammals, which traditionally has led to long production time, limited scalability and ethical concerns regarding the exposure of animals to antigens of interest. Though improved by antibody amplification technologies, such as hybridoma expression systems, these limitations are still present. Therefore, if a new assay could be developed which employed robust sensing biomolecules, which could be produced on a large scale, quickly and affordably, there would be significant scope to improve current sensing technology, not only in LOX-1 but in all other biomarkers.

Affimers are protein nanobodies which act as antibody mimetics. They are small molecules which derive their structure from plant phytocystatins and have two, variable amino acid sequences which enable binding to molecules of interest. The variation in these amino acids allows for 3×10^9 possible sequences, which in turn make a number of binding targets viable. Due to their small size and short protein sequence (12kDa), Affimers can be expressed and purified from *Escherichia Coli* which allows for low cost, high volume production and avoids any ethical concerns arising from animal experimentation.

This chapter aims to assess whether Affimers can be used to construct an assay for the detection of LOX-1 at physiological levels.

3.2 Results

3.2.1 *Expression and Purification of LOX-1 Specific Affimers*

pET 11a-plasmid vectors containing Affimers A1, B1, G1, H1 and A3 with terminal HIS and cysteine tags were transformed into XL10 E-coli cells. These five affimers were previously identified by Dr Rachel Seese while working in the Ponnambolam laboratory. In order to identify these affimers, the recombinant LOX-1 protein described by Dr Ravi Vohra and used in this thesis was screened affimer library. Sequencing (Figure 3-1A) was checked against Affimer libraries in the Tomlinson laboratory (Figure 3-1B), as described in chapter 2. After confirmation of correct sequencing, plasmid DNA was transformed into BL21 Star DE3 bacteria for protein expression. Gels were stained and the presence of protein bands at 12kDa was taken as successful purification of Affimers (Figure 3-1C).

Figure 3-1 DNA and Amino acid sequencing of Affimers
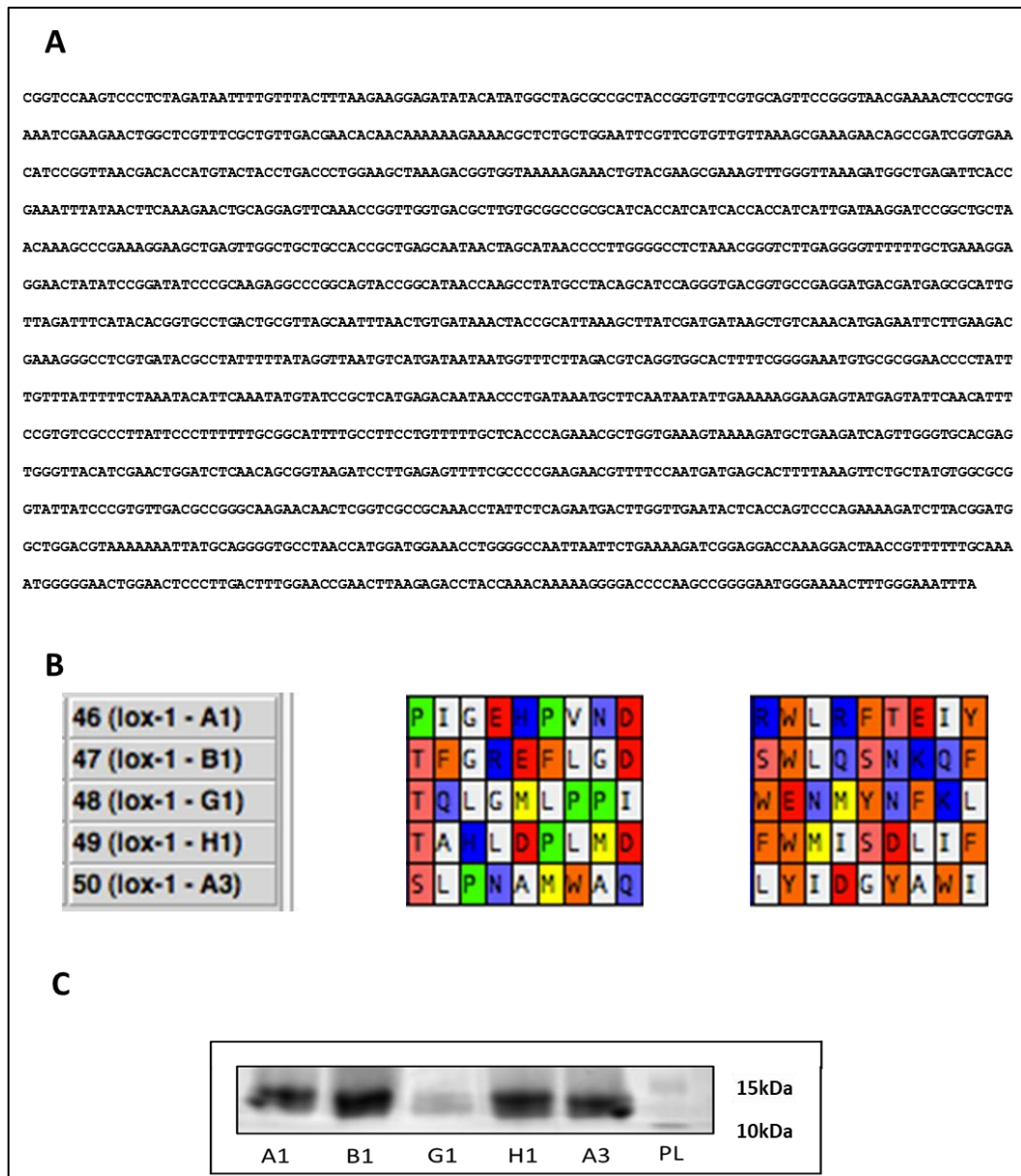
Legend: A) DNA sequence of Affimer A1. B) Amino acid sequence of variable regions of LOX-1 binding Affimers. C) Coomassie stained SDS PAGE of purified Affimer PL: Protein Ladder

3.2.2   Affimer-Based Direct ELISA

An Affimer-based direct ELISA was performed to ascertain whether Affimers could substitute antibodies for the detection of target analyte (recombinant, ECD-LOX-1). Of all five Affimers successfully screened against ECD-LOX-1, only the Affimer A1 demonstrated a statistically significant, positive correlation between colorimetry and ECD-LOX-1 concentration (r=0.9, p=0.04) (Figure 3-2). It was noted that despite this positive correlation, the overall colour change and thus recoded absorbance (Max OD_{650}=0.05) was low, even at concentrations of ECD-LOX-1 10^6-fold over recorded physiological norms. Further, though results were consistent and error margins were small, the overall increase in absorbance was within the error limits of the colorimetry hardware used. Thus, while the A1 Affimer could clearly be used for detecting ECD-LOX-1 in solution, the direct ELISA was not suitable for 'real-world' applications. In order to employ such applications, the signal generated would need to be amplified and sensitivity increased.

Figure 3-2: Single Affimer-based direct ELISAs.
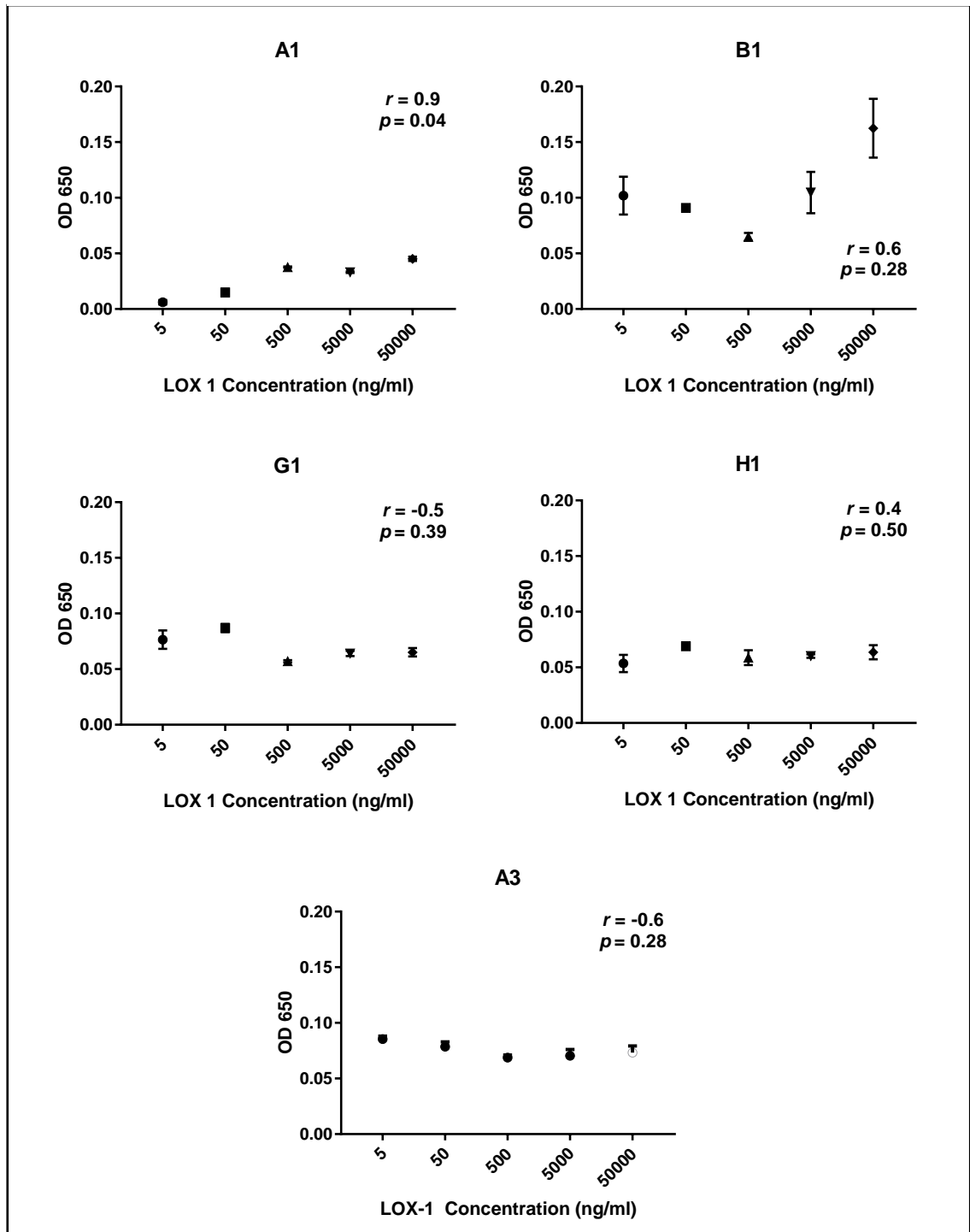
Legend: All five Affimers, conjugated to biotin were incubated with known concentrations of recombinant ECD-LOX-1. A positive correlation between concentration and colorimetry was taken as a sign of a successful ELISA. n=3. Results plotted with bars representing standard error of the mean.

3.2.4 Affimer Sandwich ELISA

All five Affimers were screened against each other in 25 possible combinations dictated by orientations of capture and detection positions. Most combinations of Affimers failed to generate a signal greater than baseline, presumably due to competitive binding sites on ECD-LOX-1. However, when combined, H1 and A1 gave a significantly higher signal than all other combinations ($p<0.0001$) (Figure 3-3A). Reassuringly, this was present in both orientations (H1 capture, A1 detection *versus* A1 capture and H1 detection), though seemed greater with H1 as the capture Affimer. These results support the notion that A1 and H1 are non-competitive ECD-LOX-1 binders

The H1/A1 Affimer sandwich ELISA was taken forward and assessed for correlation between ECD-LOX-1 and colorimetry. This showed a stronger correlation profile than the A1 direct ELISA ($r=0.98$, $p<0.0001$) (Figure 3-3B). However, the sensitivity was not convincingly improved (the lowest concentration of ECD-LOX-1 correlated with an increase in signal of 100ng/ml, still 10 000 times higher than the lowest reported physiological level). Further, the signal strength was still weak, with a peak absorbance (OD_{650}) of 0.06. Thus, to improve both signal amplitude and overall sensitivity, the H1/A1 Affimer sandwich ELISA was modified to a chemiluminescence-based platform.

Figure 3-3: Affimer Sandwich Assay Screen and Results
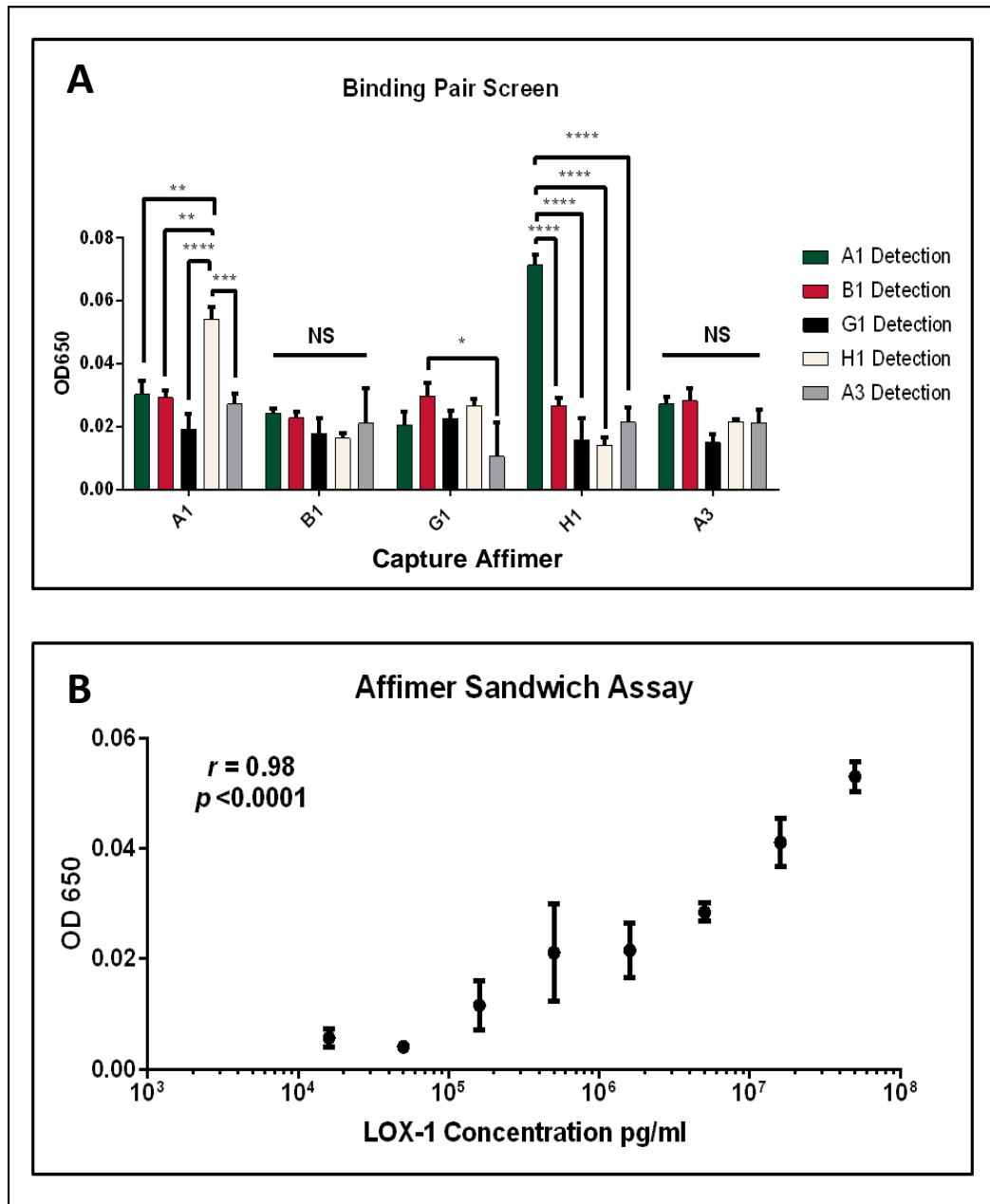
Legend: A) Sandwich binding Screen of all Affimer combinations, NS: Non-significant, OD: Optical Density. H1 / A1 Constructs had significantly higher signal levels than other combinations B) H1/A1 Affimer Sandwich ELISA. Strong positive correlation with signal strength and ECD-LOX-1 concentratoin

3.2.5   Chemiluminescence Affimer Affinity Tests

To improve sensitivity, and amplify signal, a chemiluminescence platform was adopted, as described in the methods section. The same combination of H1/A1 Affimers led to no improvement in the sensitivity of the assay, with a minimum concentration of 100ng/ml of ECD-LOX-1 being required for a detectable change in the relative light units detectable by the chemiluminescence experiment (Figure 3-4A). As a final effort develop an immunoassay-based sensing platform, a hybrid (antibody/Affimer) assay was developed. The capture/detection orientation of antibody and Affimers was tested and revealed that greatest signal was obtained when antibodies were used for capture and Affimers for detection (Figure 3-4B, C), perhaps due to the easier biotinylation of Affimers with a free cysteine group. After a screening test, similar to that outlined earlier, Affimers B1 and G1 were found to have the highest sensitivity between 0 and 500ng/ml of ECD-LOX-1. These were taken forward into the hybrid chemiluminescence platform to assess overall sensitivity. Again, the limit of detection was approximately 100ng/ml of ECD-LOX-1 (Figure 3-5), making the hybrid system no more sensitive than the Affimer-only chemiluminescence platforms.

Figure 3-4: Chemiluminescence Affimer Affinity Test
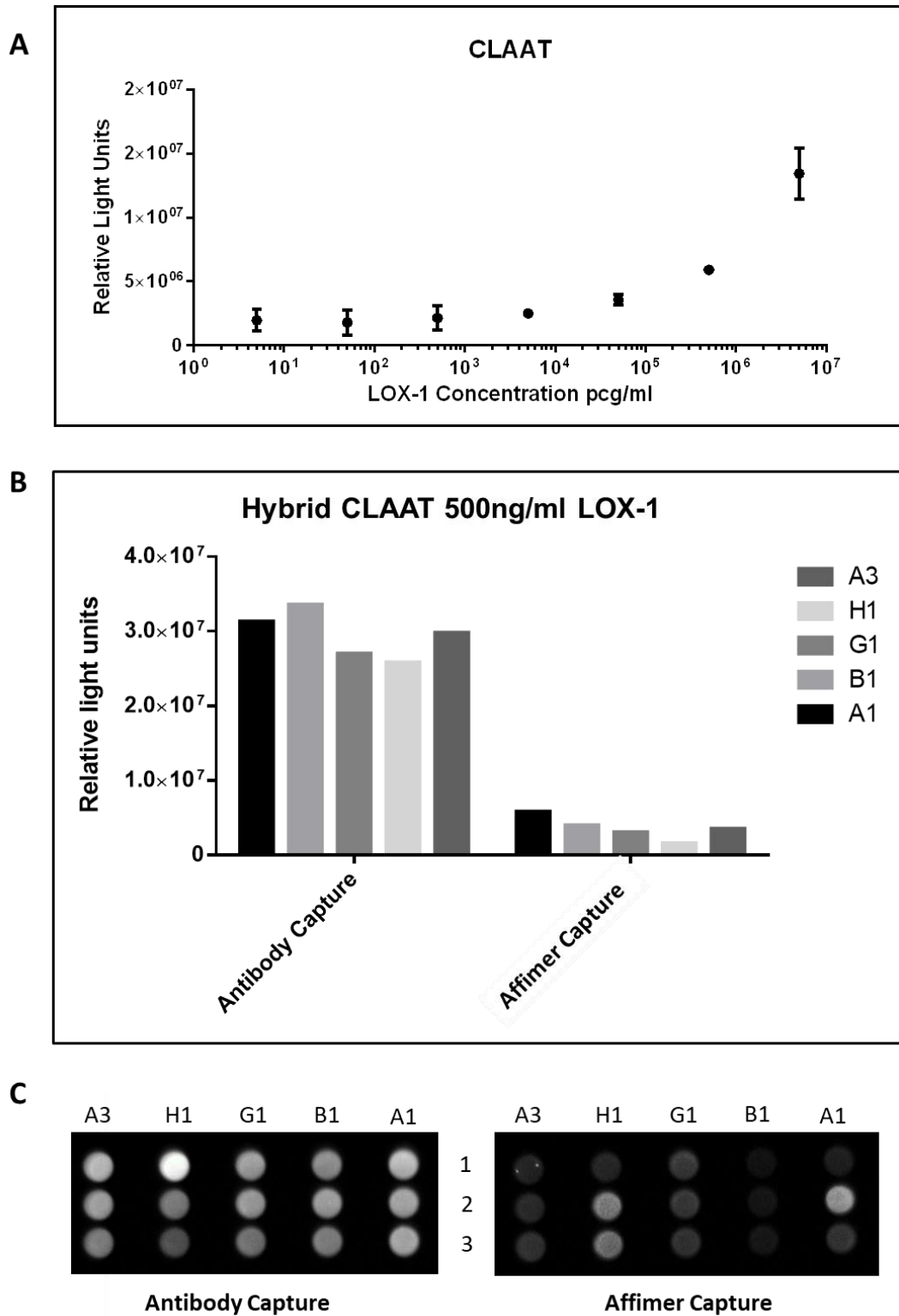
Legend: A) Affimers A1 and H1 used in combination to detect ECD-LOX-1 B) Hybrid antibody / Affimer CLAAT demonstrated significantly higher chemiluminescence when anti-LOX-1 antibody was used for immobilisation of ECD-LOX-1. C) Chemiluminescence images demonstrating difference in luminescence, numbers indicate well repetition, n=3.

Figure 3-5: Hybrid CLEIA/CLAAT optimisation
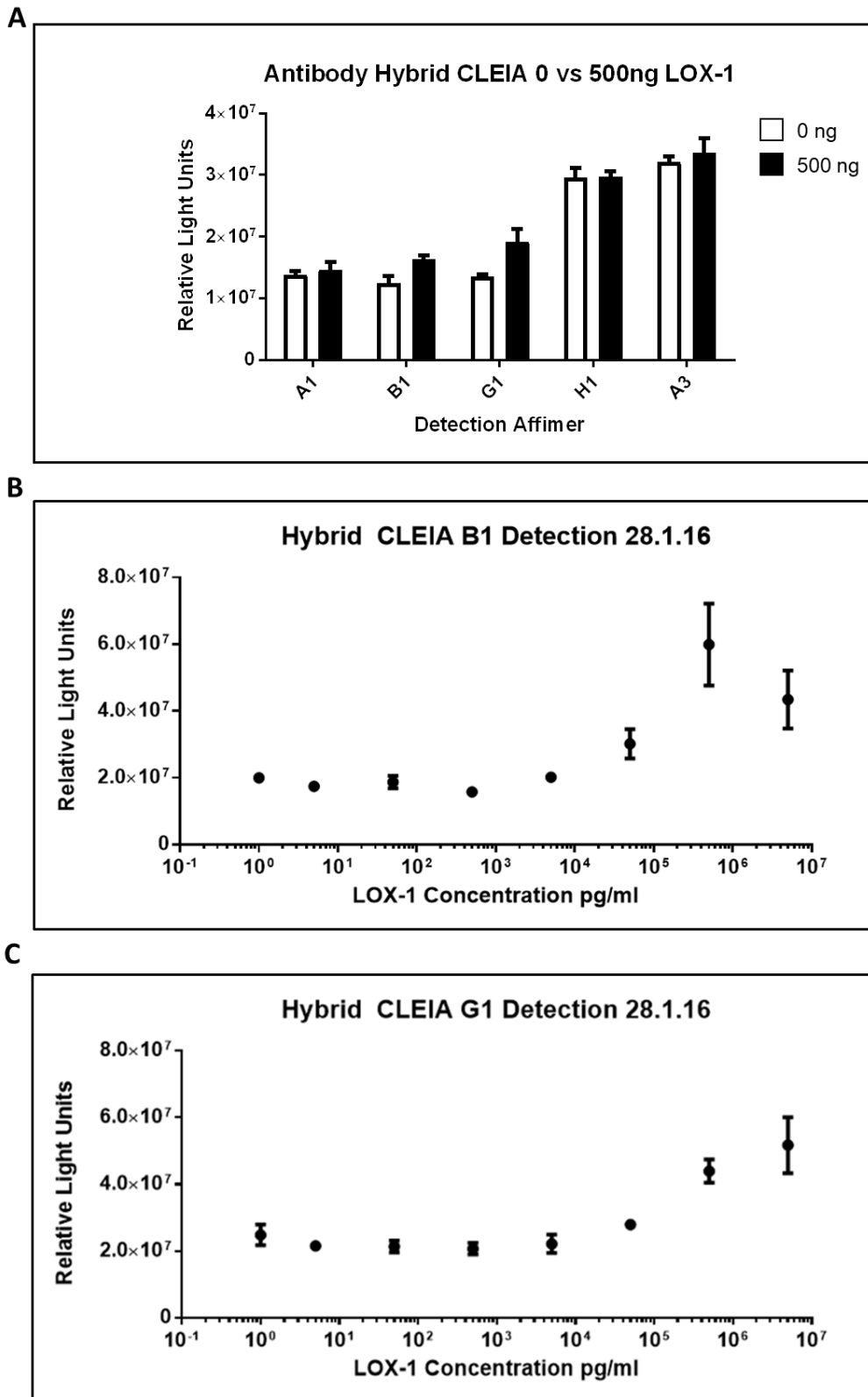
Legend: A) Testing 0 to 500ng of ECD-LOX-1 with Affimers G1 and B1 showing greatest differentiation in signal intensity. B) Antibody/B1 CLEIA demonstrates lowest detectable rise in signal at approximately 50ng ECD-LOX-1 with drop-off in signal after 500ng C) Antibody/G1 CLEIA demonstrating similar sensitivity to B1

3.2.6 Electrochemical biosensor optimisation

To this point, Affimers A1 and H1 demonstrated the strongest binding to ECD-LOX-1. For this reason, they were taken forward into biosensor construction. Two polymerisation constructs (tyramine and octopamine) were used for binding the Affimers to the electrodes, as described in the methods section.

Acid cleaning of electrodes by cyclic voltammetry revealed a change in electrode performance after the first cycle, however this was stable for subsequent cycles, denoting the possible presence of contaminants on the electrode surface from the supplier (Figure 3-6A). Polymerisation revealed a characteristic 'hump' in the voltammetry curve followed by a change in its pattern on subsequent shape on the second cycle (figure 3-6B). These characteristic changes were typical of successful polymerisation and were seen in all experimental electrodes.

Initial trials with tyramine led to extremely irregular Nyquist plots (Figure 3-6C) and uninterpretable results, perhaps suggesting that tyramine was an inadequate binding or polymerisation substrate. Octopamine plots by contrast were comparatively more uniform over fixed ranges in concentration. Next, it was clear that at frequencies below 15Hz, the Nyquist plots became very erratic. Lastly, it was observed that while there were changes in the Nyquist curve parameters between 1pg/ml and 1ng/ml, at concentrations greater than this, the curves would change unpredictably. For this reason, the frequency range was limited to 15-2500 Hz and ECD-LOX-1 was tested in concentrations of 1pg/ml-1ng/ml.

Figure 3-6: Biosensor Optimisation Steps
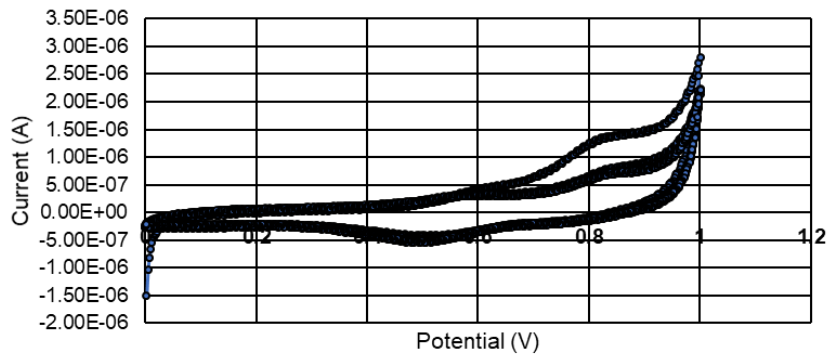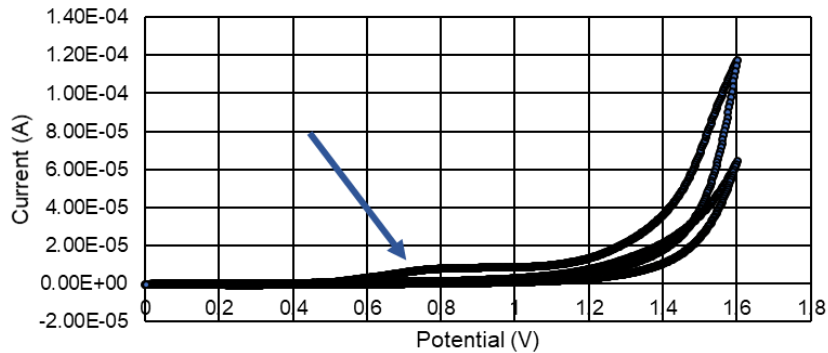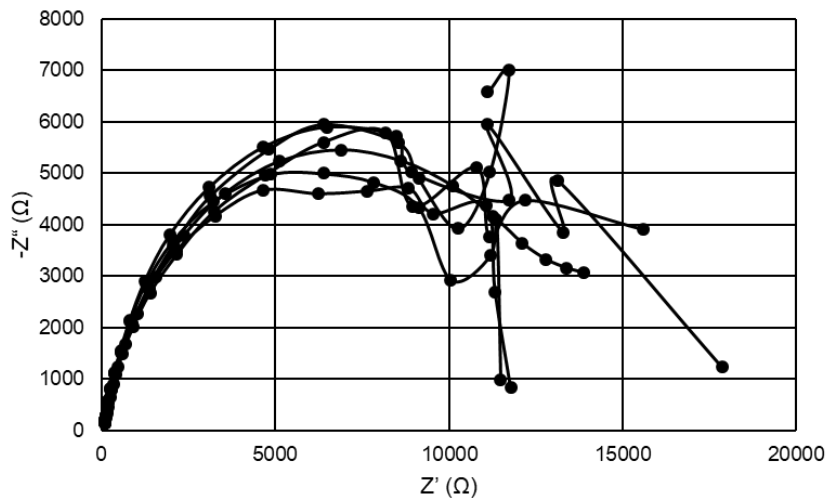
Legend: A) Acid cleaning with cyclic voltammetry led to stable electrode response. B) Characteristic curve and 'hump' (arrow) illustrating tyrosine/octopamine polymerisation on electrode C) Highly disorganised Nyquist plot using tyramine as polymerisation agent. Concentrations of LOX-1 greater than 1ng/ml and frequencies below 15Hz associated with particular derangement in plot

3.2.7 ECD-Lox-1 detection by Affimer-based biosensor

For Affimer A1, a subtle difference could be appreciated in some Nyquist plots at different LOX-1 concentrations. This difference was greatest at the lowest tested frequency (18Hz) and could be most easily appreciated as a variation in the capacitance within the circuit. However, this was an inconsistent result and could also be seen in the non-specific electrode (Figure 3-7, 3-8). Furthermore, there was great variation in the size and shape of Nyquist plots despite using the same materials, methods and conducting tests in the same environment, on the same day. The reasons for this are unclear but perhaps imply a variability in electrode surface or binding of the octopamine/Affimer conjugate. Predictably, given this variability, when plotted, resistance to charge transfer showed enormous error, even when demonstrated as a percentage change from baseline.

Ultimately, given the variability in electrode performance and the absence of a demonstrable change in circuit characteristics at different LOX-1 concentrations, it was not possible to take the A1-Affimer biosensor forward into biological sample testing.

Figure 3-7: 'Optimised' A1-based Biosensor
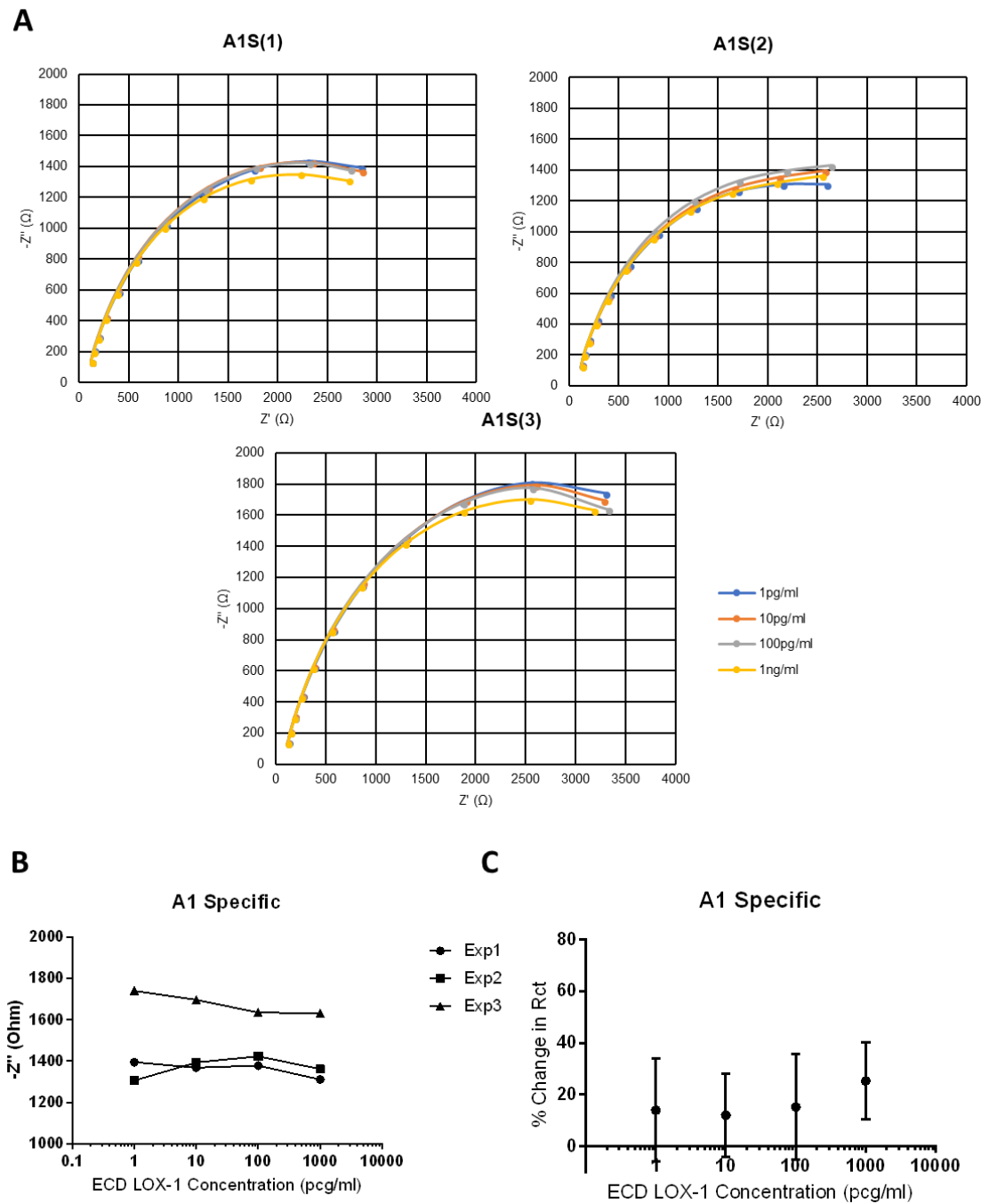
Legend: A) Organised Nyquist plots using 3 different electrode chips with A1 Affimer/octopamine polymerisation B) Capacitance response at 18Hz shows general decrease in response to higher concentration C) Generally flat percentage resistance to charge change compared to control (no ECD-LOX-1) with large error margins

Figure 3-8: GFP Affimer-Based Biosensor
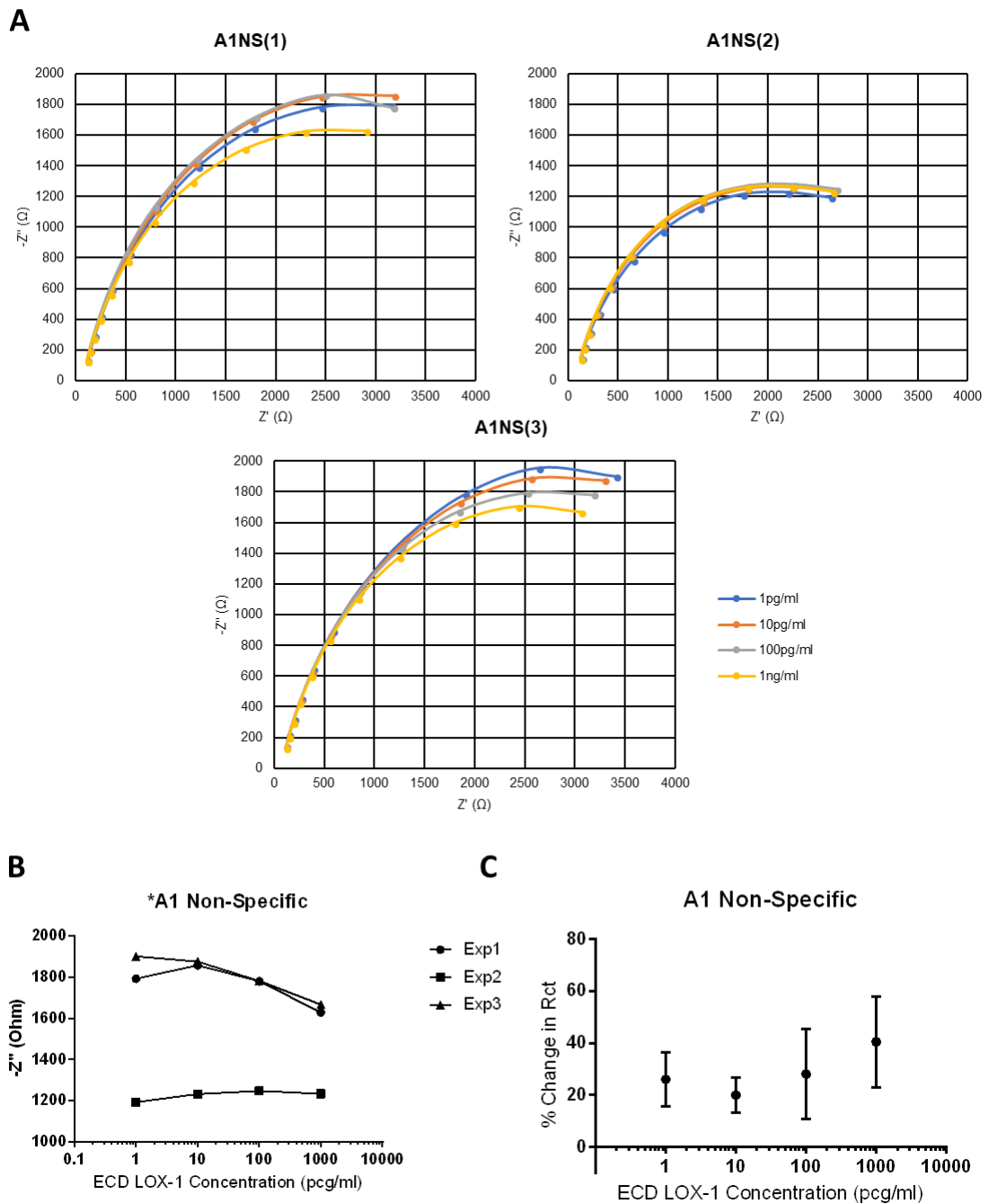
Legend: A) Nyquist plots using 3 different electrode chips with GFP Affimer/octopamine polymerisation B) Capacitance response at 18Hz shows general decrease in response to higher concentration C) Increasing percentage resistance to charge change compared to control (no ECD-LOX-1) with large error margins

The H1 Affimer displayed very similar characteristics to A1 when used in the Affimer-based biosensor. Again, there was some noticeable variation in the Nyquist plots, with a peak difference visible at the lowest tested frequency (18Hz). A notable difference was again present in the morphology of different plots, despite experiments being conducted simultaneously. However, the overall reduction in in circuit capacitance was more consistent in experiments involving Affimer H1 (Figure 3-9). Unfortunately, the same was also true in the control electrode, with the non-specific Affimer, bringing this positive finding into question (Figure 3-10). Furthermore, given that the same, non-specific Affimer was used in experiments for the A1 Affimer biosensor, it is likely that the overall reduction in capacitance is a spurious finding.

In a similar manner to the A1 Affimer experiments, H1 demonstrated a large variation in results for resistance to charge transfer across experiments, illustrated by wide error bars. Although there was a more convincing, downward trend in resistance, this was again matched by the control Affimer, making it a probable false positive. Given these results, the H1 biosensor could not be considered successful in consistently detecting variations in ECD-LOX-1 and was not taken forward for any testing of human samples.

Figure 3-9: 'Optimised' H1-Based Biosensor
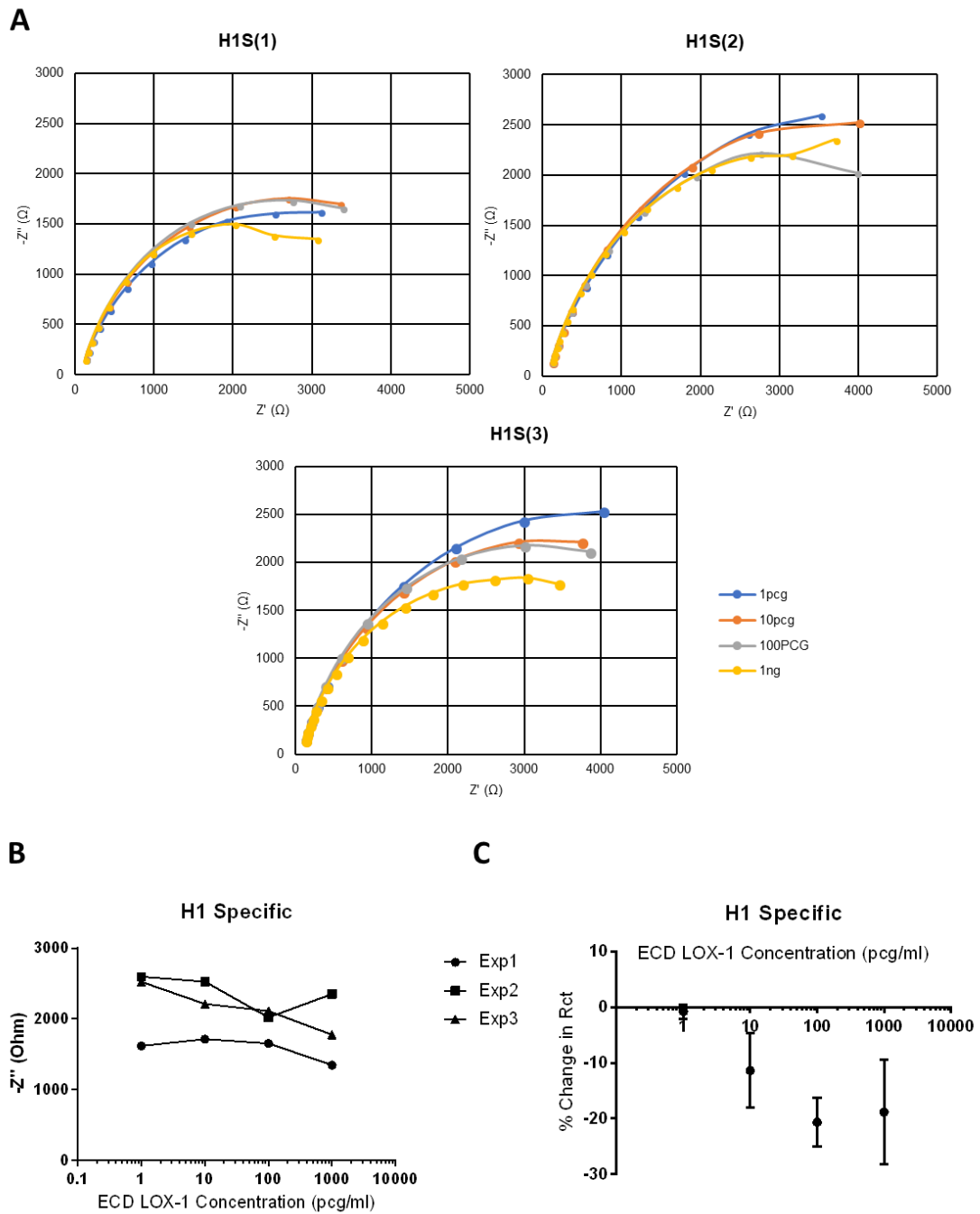
Legend: A) Organised Nyquist plots using 3 different electrode chips with H1 Affimer/octopamine polymerisation B) Capacitance response at 18Hz shows decrease in response to higher concentration C) Progressive decrease in percentage resistance to charge over 1-100pcg/ml compared to control (no ECD-LOX-1) with large error margins

Figure 3-10: GFP Affimer-based Biosensor
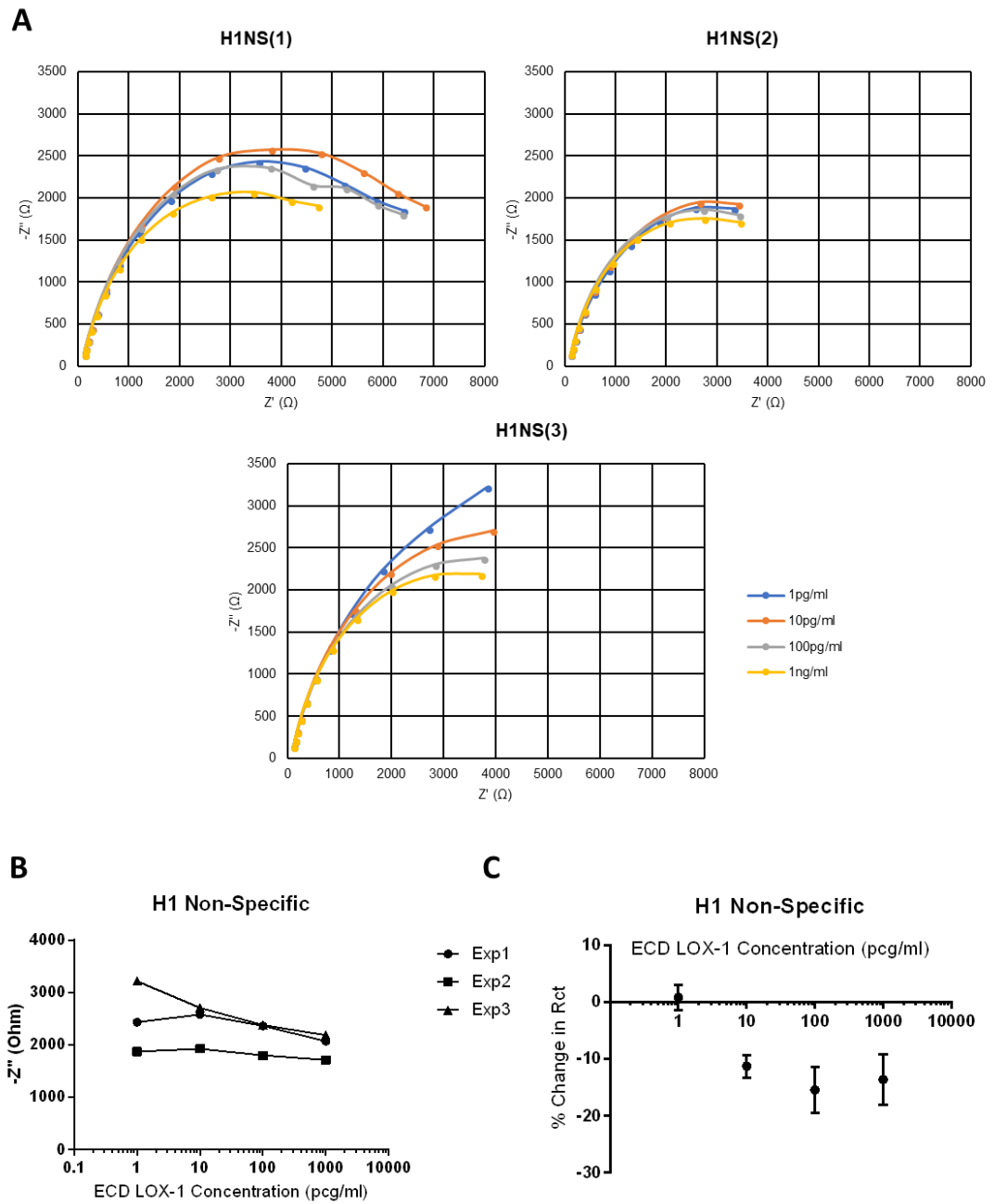
Legend: A) Nyquist plots using 3 different electrode chips with GFP Affimer/octopamine polymerisation B) Capacitance response at 18Hz shows general decrease in response to higher concentration C) Decreasing percentage resistance to charge change (1-100pcg/ml) compared to control (no ECD-LOX-1) with large error margins

3.3   Discussion

At the outset, the aim of this chapter was to develop an Affimer-based soluble LOX-1 assay or sensor. Two main techniques were employed, the first was a modified ELISA platform, including diversification into a 'sandwich' platform, chemiluminescence detection and hybrid, antibody/Affimer system. The second technique employed was the use of an electrochemical biosensor platform, binding the Affimers to organic monomers which were then polymerised onto a gold electrode and subjected to impedance spectroscopy. Attempts were also made to optimise this technique by selecting a more stable binding compound, limiting the alternating current frequencies and focusing on specific, physiologically relevant concentration ranges.

The ELISA platform proved the more successful technique. Initial results showed a weak positive correlation between colorimetry and ECD-LOX-1 concentration. However, this was not a suitable method for taking forward into experiments involving human blood samples. This was predominantly due to the fact that such experiments relied on the target being fixed on the ELISA plate. This would be challenging in a sample of human blood containing multiple proteins in higher concentration, which would presumably inhibit the binding of LOX-1 to an unprepared plate. Another consideration was that the lower limit of detection was below the physiological concentration of soluble LOX-1 reported in the literature (Kume, Noriaki et al., 2010). Screening of antibodies revealed a complimentary binding pair of Affimers. Unfortunately, although in combination, the ELISA showed much stronger signal and correlation between concentration and signal level, the

limit of detection was still too high. This was not helped by substitution with chemiluminescence-detection or the use of antibodies for LOX-1 capture. Interestingly, when used in combination with antibodies, it was Affimers G1 and B1 which demonstrated the strongest signal. Given that G1 generated no convincing signal when ECD-LOX-1 was fixed to the plate, and that the A1/H1 Affimer pair showed very little signal difference over control, it is possible the binding of the antibody to ECD-LOX-1 brings about some change in its orientation or structure, promoting, or inhibiting Affimer binding.

It was hoped that the use of a biosensor platform may lead to a more sensitive assay for ECD-LOX-1. However, despite trying to optimise the concentration range, reducing the current frequency range and trying different polymerising agents, it was not possible to demonstrate a convincing signal response in peak capacitance or charge transfer resistance after exposure to ECD-LOX-1 at increasing concentrations. The reasons for this are not immediately clear, however, of note, there was significant variation in the Nyquist plots between experiments conducted near simultaneously. Given that experiments were conducted in the same manner, with the same materials, it suggests a variability in the materials employed. In particular, the gold-coated electrodes on which the Affimers were bound, could not be quality controlled, and there may have been variation in the coating (smoothness, thickness, contaminants etc). Equally, the use of octopamine/tyramine binders in biosensor design is relatively new. Although the monomers bound the Affimers by a cysteine group in an inactive part of the molecule, it may be that the orientation of the molecule changed once the monomer had been polymerised. Equally, the response of Affimers to electrochemical forces is unknown. Though unlikely, it may be that the

very electrochemical polymerisation of the monomers led to a denaturing of the Affimers and inhibited their binding ability.

Ultimately, it has been shown that Affimers directed against ECD-LOX-1 can be successfully used for its detection by means of a modified ELISA system. However, such a system is not sensitive enough to have clinical applications. It is possible that with further work, a more sensitive platform may be developed, though to do so may require extensive investigation into the properties of Affimers in response to electrical currents, their behaviour in different polymerisation constructs and testing of different electrode 'chips' to ascertain those with the most stable electronic response.

Lastly, it is worth mentioning that neither sensing technology was validated against existing, antibody-based LOX-1 ELISA systems. This would naturally be the next step in the development of the technology and had been considered at the outset. However, neither the affimer-based ELISA, nor biosensor demonstrated sufficiently low detection limits to be of use in the sensing of physiological LOX-1 levels and as stated above, would not be used for the testing of biological samples. Thus, pragmatically, there was a futility in comparing these against existing technologies, as they were not suitable for further development.

CHAPTER 4 : THE LOX-1 SCAVENGER RECEPTOR AS A TARGET FOR AFFIMERS

4.1 Introduction

The lectin-like oxidised low-density lipoprotein receptor-1 (LOX-1, OLR1) is found on the surface of endothelial cells, smooth muscle cells, macrophages and platelets, among others. As its name suggests, its discovery by Tatsuya Sawamura, in 1997 focused on how modified low-density lipoproteins are recognised by endothelial cells (Sawamura et al., 1997). The accumulation of oxidised lipids in the sub-intima of arterial walls is understood to be a critical step in the formation of atherosclerosis, a pre-morbid state leading to ischaemic heart disease, strokes, mesenteric angina and peripheral arterial disease(Di Pietro et al., 2016). Thus, it is hypothesised that if oxLDL- specific membrane transporters could be modulated, then atherosclerosis may be prevented.

In line with the above hypothesis, LOX-1 null (-/-) mice have been shown to have reduced atherogenesis(Mehta, J. L. et al., 2007) and mice injected with viral vectors promoting over-expression of LOX-1, directly into the carotid artery, have greater atherogenesis(White et al., 2011). However, while such experiments serve to lend support to the idea that LOX-1 has an important role in atherogenesis, they do not provide a viable therapeutic option for the prevention of atherosclerosis. The inhibition of LOX-1 has been studied in vitro, using TS20, a LOX-1-specific monoclonal antibody. It has been shown that TS20 can reverse the impairment of vascular relaxation, brought about by LOX-1 (English et al., 2013), other studies analysing LOX-

1 inhibition have demonstrated a reduction in carotid neointimal hyperplasia, renal artery disease and ischaemic infarction.

To date, no studies have attempted to demonstrate a reduction in atherosclerosis in humans, as a result of LOX-1 inhibition. This may in part be due to the technical and ethical limitations of offering subjects viral gene therapy or antibody therapy in the long term to prevent or stabilise a disease which takes decades to develop. Nonetheless, preventative monoclonal antibody therapy for cardiovascular disease has begun to emerge, over the last decade. However, such treatments are enormously expensive and are used under relatively limited indication at present (National Institute for Health and Care Excellence, 2016). Thus, an alternative approach to inhibiting LOX-1 may be warranted and new, small-molecule inhibitors are under investigation(Thakkar et al., 2015).

Affimers are small synthetic proteins and showing increasing use in a diverse range of applications. Affimers specific for LOX-1 may provide a viable alternative to antibody inhibition of the receptor. As discussed earlier, they have the advantage of being small molecules (10kDa) which are easily produced in bacterial hosts, and thus can be generated in large volumes at relatively low cost(Tiede, C. et al., 2017).This chapter aims to assess whether Affimers can be used to successfully inhibit the LOX-1 cell receptor and thus prevent the uptake of oxLDL by endothelial cells.

4.2   Results

4.2.1   *Oxidation of LDL and fluorescent labelling of oxLDL*

Low density lipoprotein particles were purified from the blood of healthy human volunteers using established procedures (see Materials and Methods). Successful oxidation of LDL and conversion to oxLDL using incubation with copper sulphate was confirmed by agarose gel electrophoresis and staining with the dye Sudan black (Fig. 4.1A). Two different lipophilic fluorescent dyes were tested for labelling of oxLDL particles: Di-I and Di-D. The purified, fluorescent labelled oxLDL particles were incubated with HEK-293T cells, expressing human LOX-1 (see Materials and Methods). Inducible expression of FLAG-tagged human LOX-1 in HEK-293T cells was evident in both experiments (Fig. 4.1B). Incubation of HEK-293T LOX-1-expressing cells with Di-I-labelled oxLDL did not reveal any evidence of binding or uptake of these particles upon excitation and emission at the Di-D-specific wavelength (Fig. 4.1B, upper panel). However, Incubation of HEK2-93T LOX-1-expressing cells with Di-I-labelled oxLDL showed substantial red fluorescence upon excitation and emission at the Di-I-specific wavelength (Fig. 4.1B, lower panel). Thus only Di-I-labelled oxLDL particles can be specifically recognised and bound by the LOX-1-FLAG protein in this cellular assay.

Figure 4-1: LDL processing and oxLDL analysis.
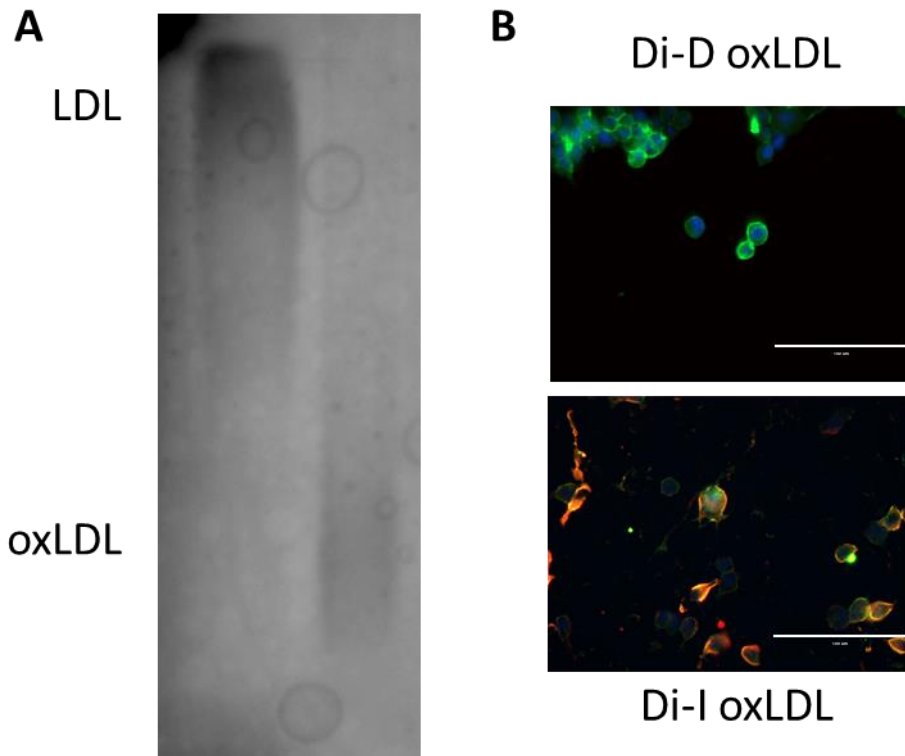
Legend: (A) Distinct bands of purified LDL and oxLDL particles are visible by agarose electrophoresis and Sudan black staining (see Materials and Methods). (B) Different lipophilic dyes were tested for labelling of oxLDL particles and binding by HEK293T cells expressing LOX-1-FLAG (green). No demonstrable red fluorescence visible with Di-D labelled oxLDL, but Di-I-labelled oxLDL is detected. . Blue: DAPI, Green: FLAG, Red: Di-D/Di-I. Scale bar denotes 100micrometres

4.2.2   Inducible LOX-1 Expression in HEK-293T cells

The tetracycline-inducible expression of LOX-1-FLAG in human HEK-293T cells stably expressing a cDNA construct was demonstrated by immunoblotting. As predicted, HEK-293T cells transfected with an empty plasmid vector (EV) had a very low level of signal when blotted with the anti-LOX-1 antibody. In contrast HEK-293T cells transfected with a LOX-1-FLAG plasmid vector (LOX-1) and induced with 1 μg/ml tetracycline for 16-20 h showed clear and significant presence of LOX-1 protein. Using tubulin as a loading control, the difference in inducible expression for LOX-1 was highly significant (**, $p<0.01$). (Figure 4.2A)

The LOX-1 vector construct also included a FLAG protein tag to facilitate blotting with a commercially available anti-FLAG antibody. Immunostaining with anti-FLAG antibody also revealed a clear difference in LOX-1 expression. Importantly, the binding of Di-I stained ox-LDL was also visibly lower in the negative control EV cell line. This experiment served to illustrate that HEK-293T cells did not natively bind oxLDL in the timeframe proposed, and thus such activity was dependent on the activity of the inducible expression of LOX-1, confirming that this is a useful cellular model for studying LOX-1 function and properties. (Figure 4.2B)

Figure 4-2: Characterisation of an inducible LOX-1-specific HEK-293T expression system
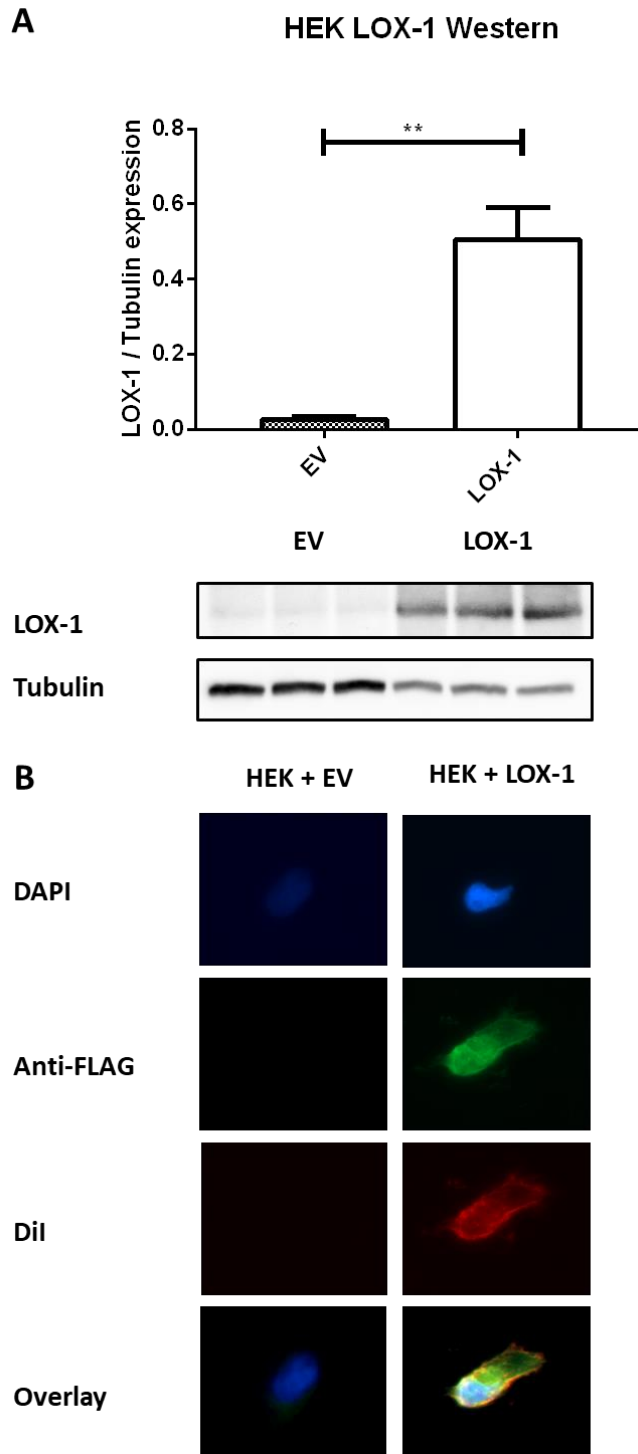
Legend: (A) Immunoblotting of control (EV) and LOX-1-FLAG expressing (LOX-1) HEK-293T cells after treatment with tetracycline overnight. Statistical analysis of LOX-1 expression (upper panel)based on immunoblot data (lower panel). Error bars denote $\pm$SEM; **, $p<0.01$. . (B) Immunofluorescence analysis demonstrates both anti-FLAG staining and oxLDL uptake in LOX-1-FLAG expressing cells but none in the control EV cell line

4.2.3   Affimer-mediated inhibition of oxLDL binding to LOX-1-expressing HEK293T

Cells

These studies established that binding of DI-I-labelled oxLDL particles to HEK-293T cells, was dependent on the presence of the LOX-1-FLAG protein (Fig. 4.3A). Using this assay, cells were pre-incubated for 5 minutes with increasing concentrations (50-500 000 pg/ml) of Affimers A1, B1, G1, H1 and A3 (Fig. 4.3B), followed by incubation with 10 mcg/ml of Di-I-labelled oxLDL for 30 minutes. Fluorescence microscopy revealed marked reduction in Di-I-labelled oxLDL binding at maximum concentration (500 ng/ml) of Affimers A1, B1 but not G1 (Fig. 4.3B). Further analysis confirmed that Affimer A3 and H1 also appear to inhibit Di-I-oxLDL binding and uptake (Fig. 4.4A). When subjected to digital quantification of image datasets, the relative reduction in Di-I-labelled oxLDL staining of LOX-1-expressing cells (positive control) and same cells treated with the specific Affimers was highly significant ($p<0.0001$; Fig. 4.4B. Thus Affimers A1, A3, B1 and H1 appear to inhibit Di-I-labelled oxLDL binding and uptake by LOX-1-expressing HEK-293T cells.

By contrast, the Affimer G1 appeared to act in a different manner by increasing Di-I-oxLDL binding to LOX-1-expressing cells (Fig. 4.3B). Quantification of Affimer G1-mediated effects also revealed that Di-I-oxLDL labelling of LOX-1-expressing cells to be significantly higher than in positive controls ($p<0.0001$; Fig. 4.4B). Thus Affimer G1 appears to promote Di-I-labelled oxLDL binding and uptake by LOX-1-expressing HEK-293T cells.

Figure 4-3: Perturbation of Di-I-oxLDL binding to LOX-1-expressing cells by Affimers A1, B1 and G1
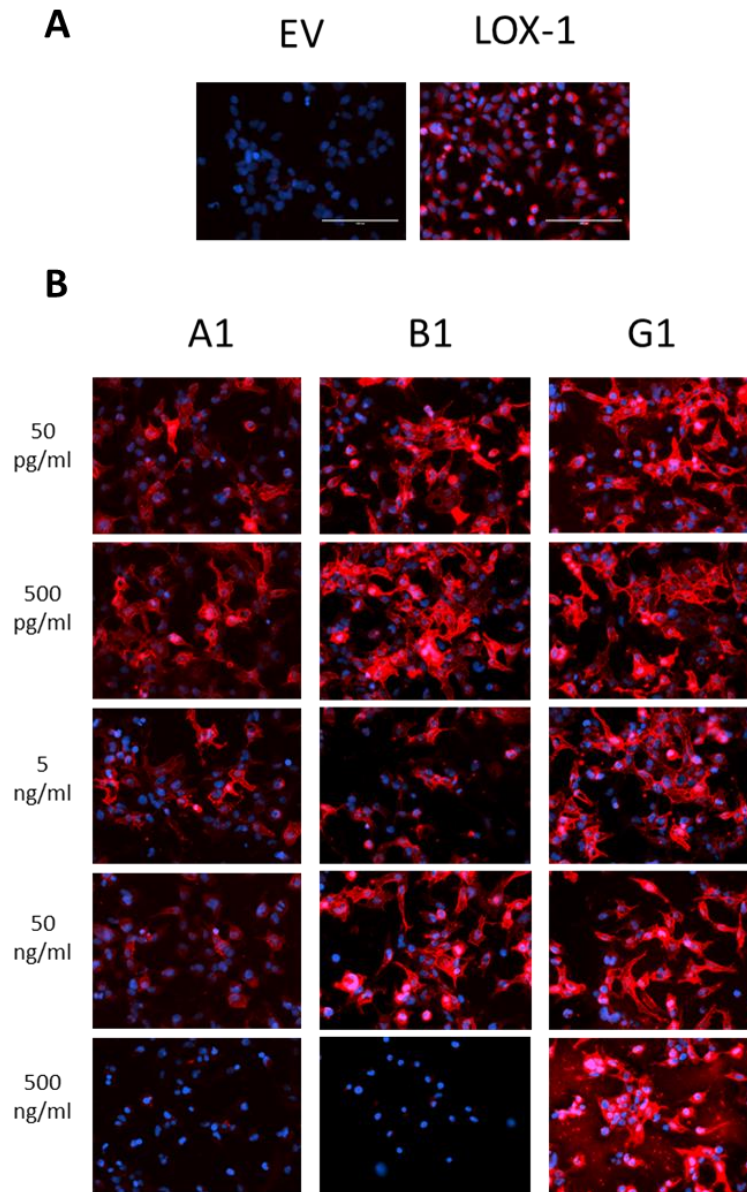
Legend: (A) Binding of Di-I-oxLDL particles to LOX-1-expressing cells, but not the negative control (EV) (Scale Bar 100um). (B) Titration of increasing Affimer (0-500 ng/ml) and evaluation of Di-I-oxLDL binding in LOX-1-expressing cells. Note that A1 and B1 show different effects compared to G1. Nuclear stain, DAPI (blue) and oxLDL (red).

Figure 4-4: Perturbation of Di-I-oxLDL binding to LOX-1-expressing cells by Affimers H1 and A3
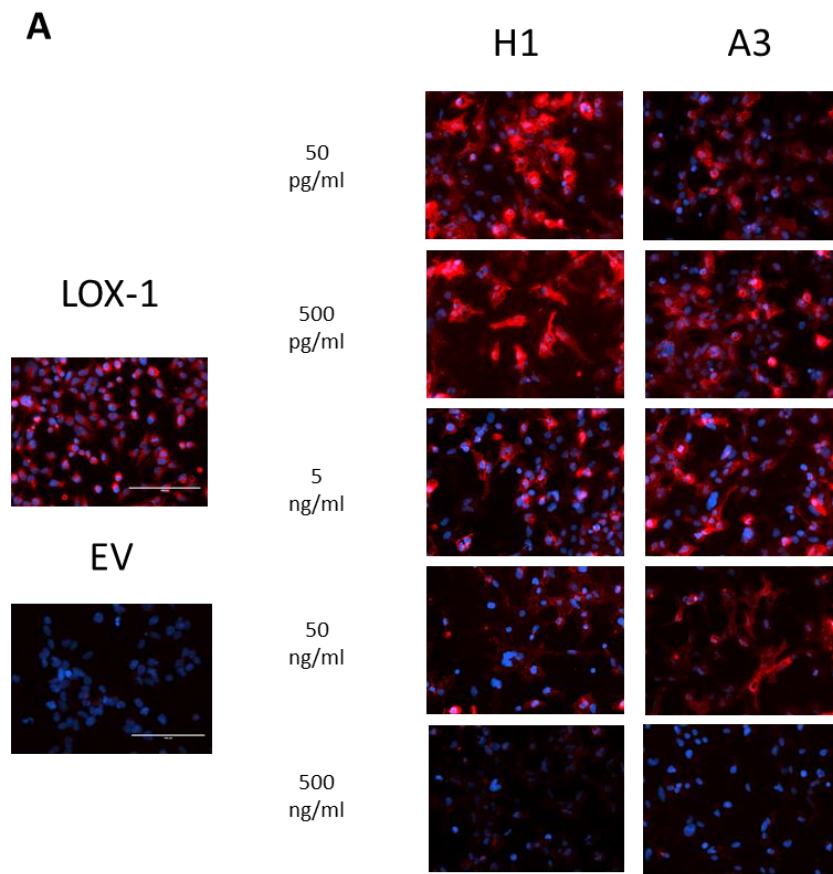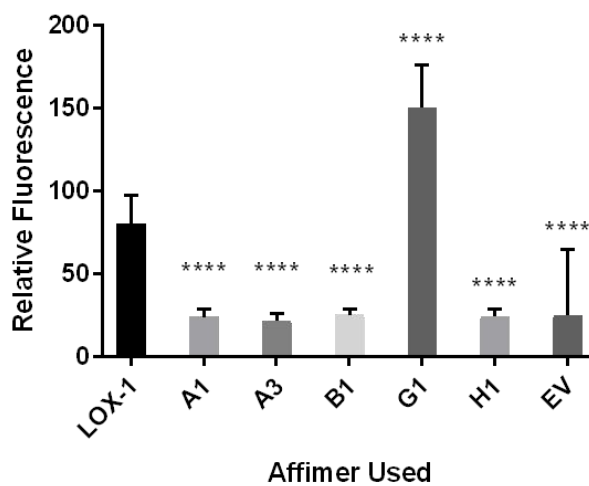
Legend (A) Binding of Di-I-oxLDL particles to LOX-1-expressing cells, in the presence of increasing amounts of Affimer H1 or A3. Scale Bar 100um(B) Quantification and comparison of Di-I-oxLDL surface binding in LOX-1-expressing cells at maximal Affimer concentration (500 ng/ml). Error bars: +SEM; All groups demonstrate reduced fluorescence compared to LOX-1 positive controls:****, p<0.0001.

4.2.4 Affimer-based perturbation of oxLDL endocytosis and LOX-1 recycling in HEK-293T cells

It was unclear whether these Affimers could modulate the trafficking of oxLDL and LOX-1 in this cellular model. To test this idea, a series of experiments were carried out to assess this using this inducible LOX-1-FLAG expression system. The HEK-293T-EV cell line was used as a negative control for assess baseline oxLDL uptake and staining for LOX-1. Di-I-oxLDL uptake was visible in LOX-1 transfected cells at 30 min of incubation, though not in the EV control (Fig. 4.5A). At 60 min, Di-I-oxLDL staining could be detected in control EV cells, suggesting that oxLDL could be bound and accumulated by a human LOX-1-independent pathway (Fig. 4.5A). However, Di-I-oxLDL staining was generally much higher in LOX-1-expressing HEK-293T cells (Fig. 4.5A). At 180 min, both negative control and LOX-1-expressing cell lines showed marked accumulation of Di-I-oxLDL (Fig. 4.5A). Quantification of digital images of these fluorescent cell profiles showed that LOX-1-expressing cells demonstrated significantly greater accumulation of oxLDL after 180 min incubation period (p=0.02; Fig. 4.5B).

Using this finding as a basis for testing the effects of Affimers on oxLDL and LOX-1 trafficking and accumulation, we pre-incubated negative control (EV) or LOX-1-expressing cells with Affimers, followed by a 180 min incubation with Di-I-oxLDL (Fig. 4.6). The findings showed significant variability, even within the same experiment (Fig. 4.6A). Generally, Affimers A1 and A3 exhibited significant reduction in Di-I-oxLDL binding and uptake over 180 min (p<0.01; Fig. 4.6B). In contrast to earlier findings, di-I-oxLDL uptake and accumulation appears to be promoted by Affimer H1 (p<0.001; Fig. 4.6B).

The above findings suggest that the uptake and recycling of oxLDL in HEK293T cells is mediated by other receptors during the period between 15 and 180 minutes, therefore nulling the effect of the inducible LOX-1 system. Such receptors are also seemingly not inhibited by the presence of Affimers which are directed towards LOX-1, suggesting that they are distinct from the receptor studied herein. Thus, to assess whether affimers may have a sustained effect on vascular tissues, a native vascular endothelial cell was studied next.

Figure 4-5: Di-I-oxLDL accumulation within LOX-1-expressing cells
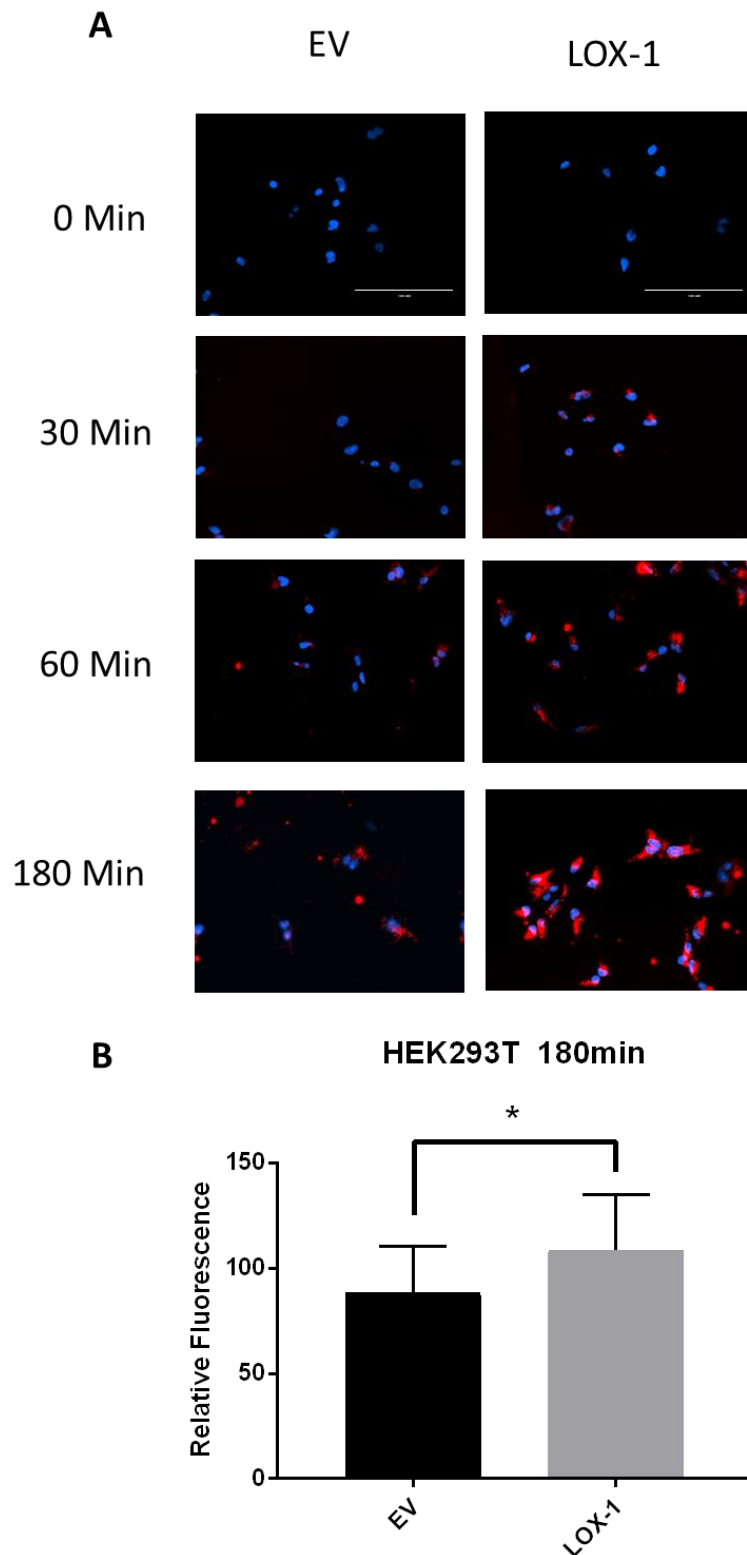
Legend: (A) Binding of Di-I-oxLDL particles to negative control (EV) or LOX-1-expressing cells.. Nuclear stain, DAPI (blue); Di-I-oxLDL (red). Scale Bar denotes 100micrometres (B) Quantification of Di-I-oxLDL binding in negative control (EV) vs. LOX-1-expressing cells. Error bars indicate +SEM; *, $p < 0.05$

Figure 4-6: Di-I-oxLDL accumulation within LOX-1-expressing cells in the presence of Affimers.
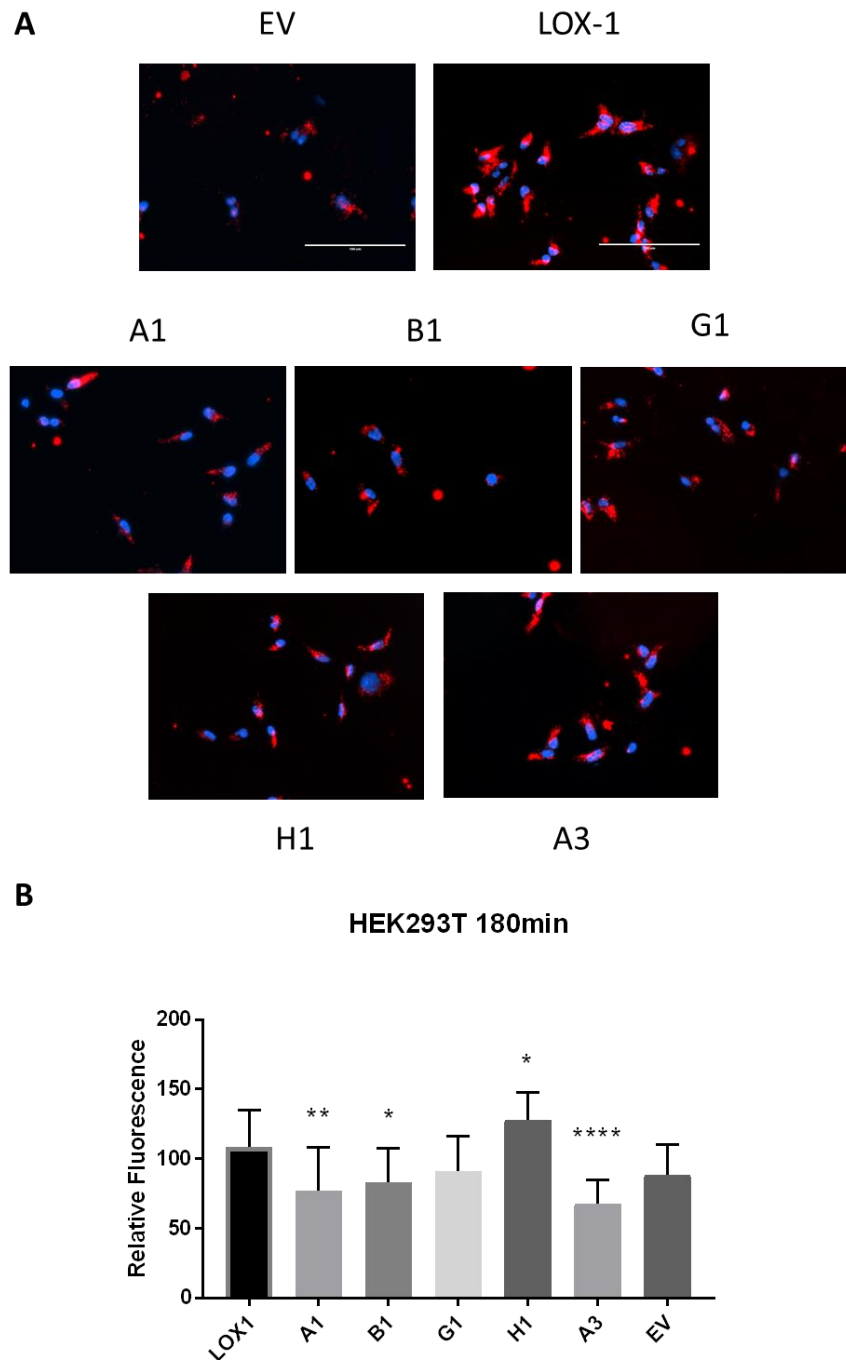
Legend: (A) Binding of Di-I-oxLDL particles to negative control (EV) or LOX-1-expressing cells in the presence of Affimer (500 ng/ml). Scale bar, 100 um. Nuclear stain, DAPI (blue); Di-I-oxLDL (red). (B) Quantification and comparison of Di-I-oxLDL binding in positive control LOX-1-expressing cells vs. Affimer-treated LOX-1-expressing cells. Error bars indicate +SEM; * p<0.05, **p<0.01. ****p<0.0001

4.2.5   LOX-1 Expression in a Porcine Aortic Endothelial Line

One caveat of the previous studies is that the human embryonic kidney cell line HEK-293T although experimentally tractable, is relatively non-physiological in relation to vascular properties such as signal transduction and angiogenesis. Another alternative was to express LOX-1-FLAG in a vascular endothelial cell line. Although these are relatively few, the porcine aortic endothelial cell (PAEC) line has been used in a number of endothelial studies (Arbogast et al., 1982; Boccafoschi et al., 2005; Basini et al., 2007). A tet-inducible PAEC-LOX-1-FLAG model was provided and tested (Fig. 4.7). Immunoblot analysis showed statistically significant expression of LOX-1 (p<0.001) based on immunoblot data comparing a negative control (EV) and LOX-1-expressing line (Fig. 4.7A). There was concern that this endothelial cell line may have native (porcine) LOX-1 expression, however PAECs transfected with an empty vector (EV) had a very low signal when blotted with the anti-(human) LOX-1 antibody, though the cross-species reactivity for this sheep anti-human LOX-1 antibody is not known.

Like in HEK293Ts, the LOX-1 vector construct included a FLAG protein tag to facilitate blotting with anti-FLAG antibody. Immunostaining with anti-FLAG antibody also revealed a clear signal for LOX-1 expression. However, there was visible binding and accumulation of Di-I-oxLDL in the negative control (EV) (Fig. 4.7B). Furthermore, the binding of Di-I-oxLDL to LOX-1-FLAG-expressing PAEC cells appeared at first sight to be similar to that of EV-expressing PAECs (Fig. 4.7B). These findings suggested that LOX-1-FLAG expression in the PAEC line might not contribute to the binding of oxLDL in isolation.

Figure 4-7: Characterisation of an inducible LOX-1-specific PAEC expression system.
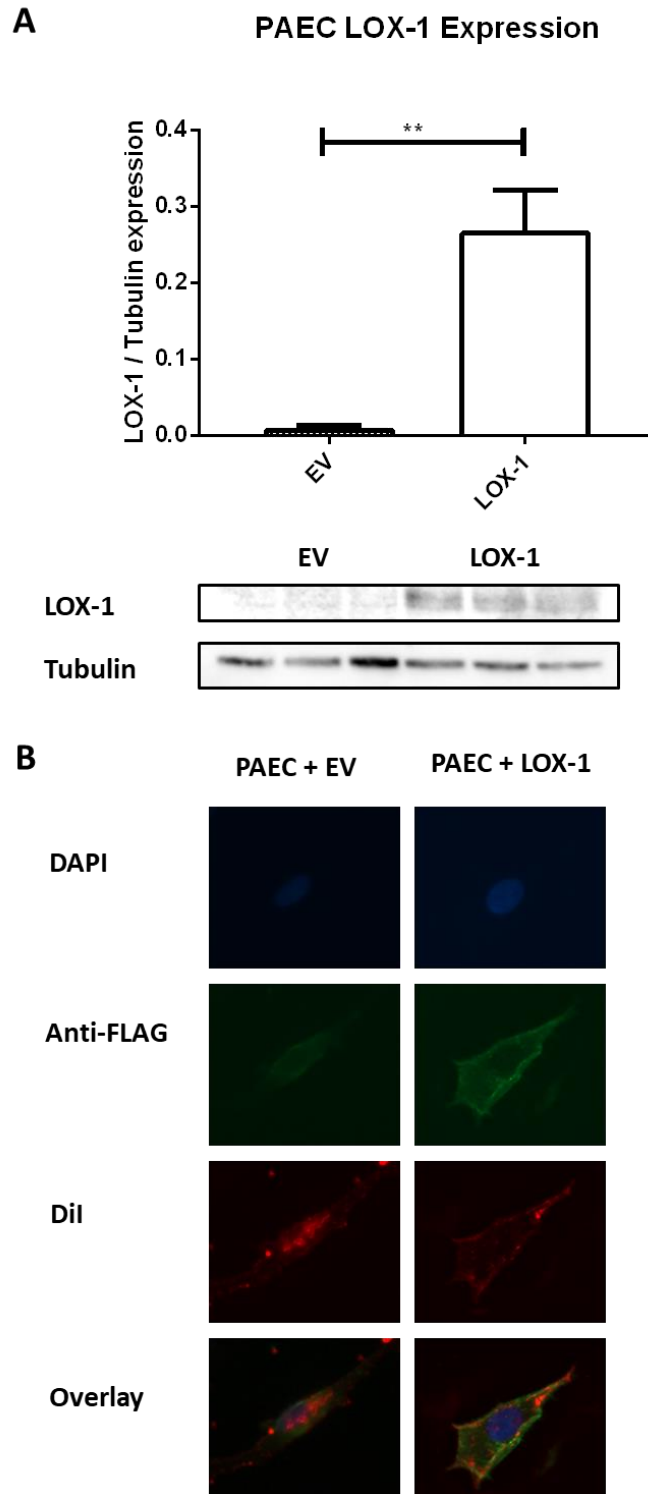
Legend: (A) Immunoblotting of control (EV) and LOX-1-FLAG expressing (LOX-1) PAEC cells after treatment with tetracycline overnight. Statistical analysis of LOX-1 expression (upper panel) based on immunoblot data (lower panel). Error bars denote +SEM; **, p<0.01. (B) Immunofluorescence analysis demonstrates anti-FLAG staining and Di-I-oxLDL staining in cells stained with DAP (blue).

4.2.6   Affimer- mediated inhibition of oxLDL binding to LOX-1-expressing PAECs

Despite the presence of Di-I oxLDL staining in EV-PAEC lines, when subjected to digital quantification of image datasets, the difference in Di-I-oxLDL staining between LOX-1 and EV-transfected PAECs was found to reach statistical significance ($p<0.0001$; Fig. 4.8B). This made the assessments of Affimers as inhibition agents feasible. Despite the fact that the EV cell line bound Di-I oxLDL, it could still serve as a negative control, given that it was statistically different from the LOX-1-expressing PAEC line.

The pre-incubation of PAECs with Affimers led to a dramatic reduction in Di-I-oxLDL staining using fluorescence microscopy (Fig. 4.8A). Quantification of image datasets demonstrated that this was highly significant for all Affimers when compared to the untreated LOX-1 cell line, even when accounting for multiple analysis correction ($p<0.0001$ for all comparisons; Fig. 4.8B). Of note, LOX-1-transfected cells which were pre-incubated with Affimers demonstrated a lower overall fluorescence than negative control (EV) PAECs which had not been pre-incubated. When images from Affimer containing cells were compared against EV cell line wells, this reached statistical significance for all Affimers except for Affimer A1.

Figure 4-8: Di-I-oxLDL accumulation within LOX-1-expressing PAECs in the presence of Affimers.
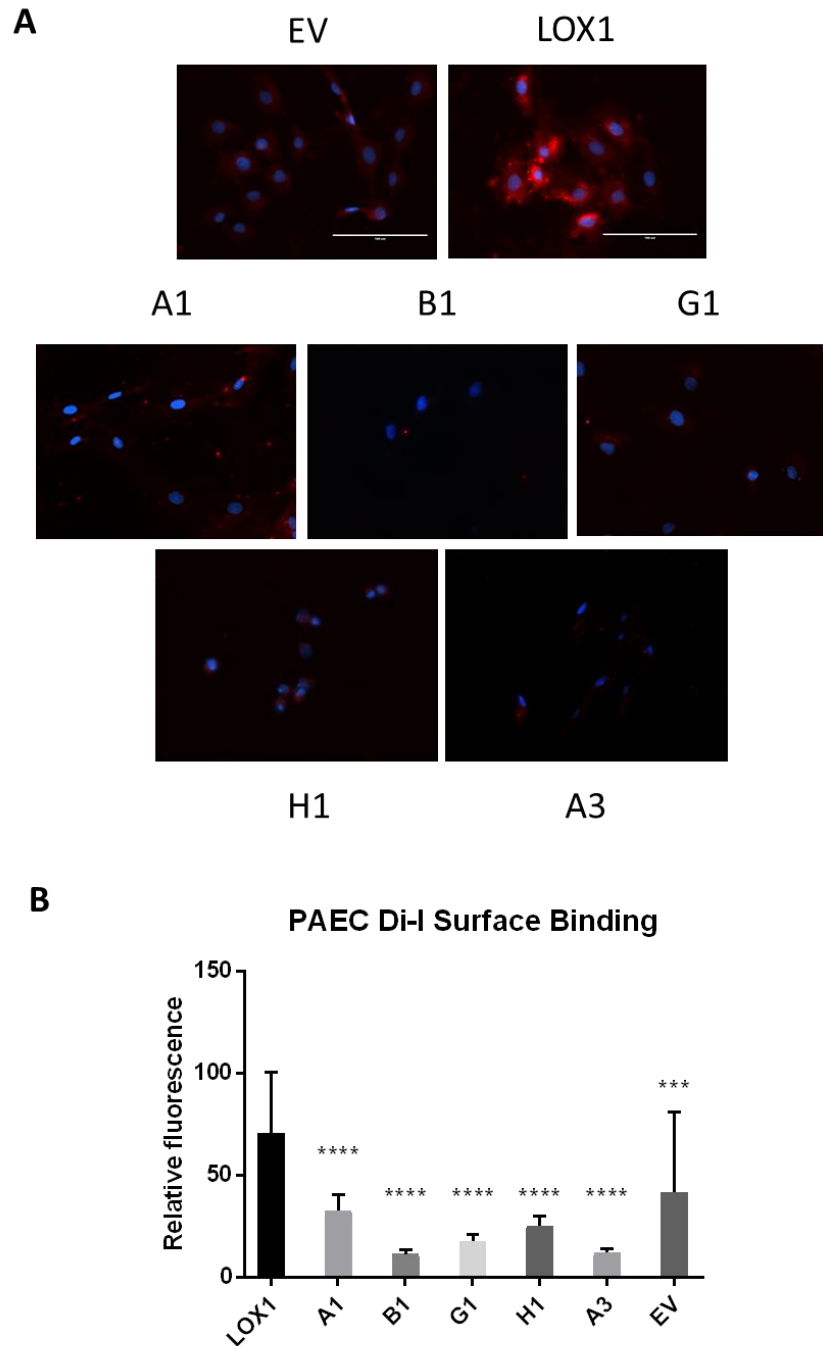
Legend: (A) Binding of Di-I-oxLDL particles to negative control (EV) or LOX-1-expressing cells in the presence of Affimer (500 ng/ml). Nuclear stain, DAPI (blue); Di-I-oxLDL (red). Scale bar, 100 um. (B Quantification and comparison of Di-I-oxLDL surface binding in LOX-1-expressing cells at maximal Affimer concentration (500 ng/ml). Error bars indicate +SEM; All groups demonstrate significant reduced fluorescence compared to LOX-1 positive controls: ***,p<0.001; ****, p<0.0001

4.2.7 Affimer-mediated perturbation of oxLDL binding and accumulation by LOX-1-expressing PAECs

Prolonged incubation of PAECs with oxLDL demonstrated that the EV cell line reached saturation by 120 min post-incubation with Di-I-oxLDL (Fig. 4.9A). In contrast to short incubation periods on ice, the difference in Di-I-oxLDL binding and accumulation between negative control (EV) and LOX-1 expressing PAECs after 120 min was not visually evident. Despite the lack of di-I-oxLDL accumulation in the EV and LOX-1 cell lines, the effect of Affimers was still assessed, given that they had previously demonstrated inhibition of oxLDL binding above the level of the empty vector cell line. Only Affimer H1 seemed to significantly inhibit oxLDL staining ($p<0.0001$; Fig. 4.9B), whereas Affimers B1 and A3 significantly boosted oxLDL accumulation ($p<0.001$; Fig. 4.9B). The effects caused by other Affimer tratments was not significant in this assay.

Of note, the overall uptake of Di-I-oxLDL in PAECs was highly heterogenous across all wells after 120 min incubation period. Some clusters of cells would appear completely saturated, while others would seem to have very little uptake, if any. Thus, it was difficult to gain much certainty as to whether the results were a true reflection of the effects of affimers, or, perhaps more likely, discrepancies in image capture brought about by a heterogenous sample.

**Figure 4-9: Sustained Di-I-oxLDL accumulation within LOX-1-expressing PAECs in the presence of Affimers**
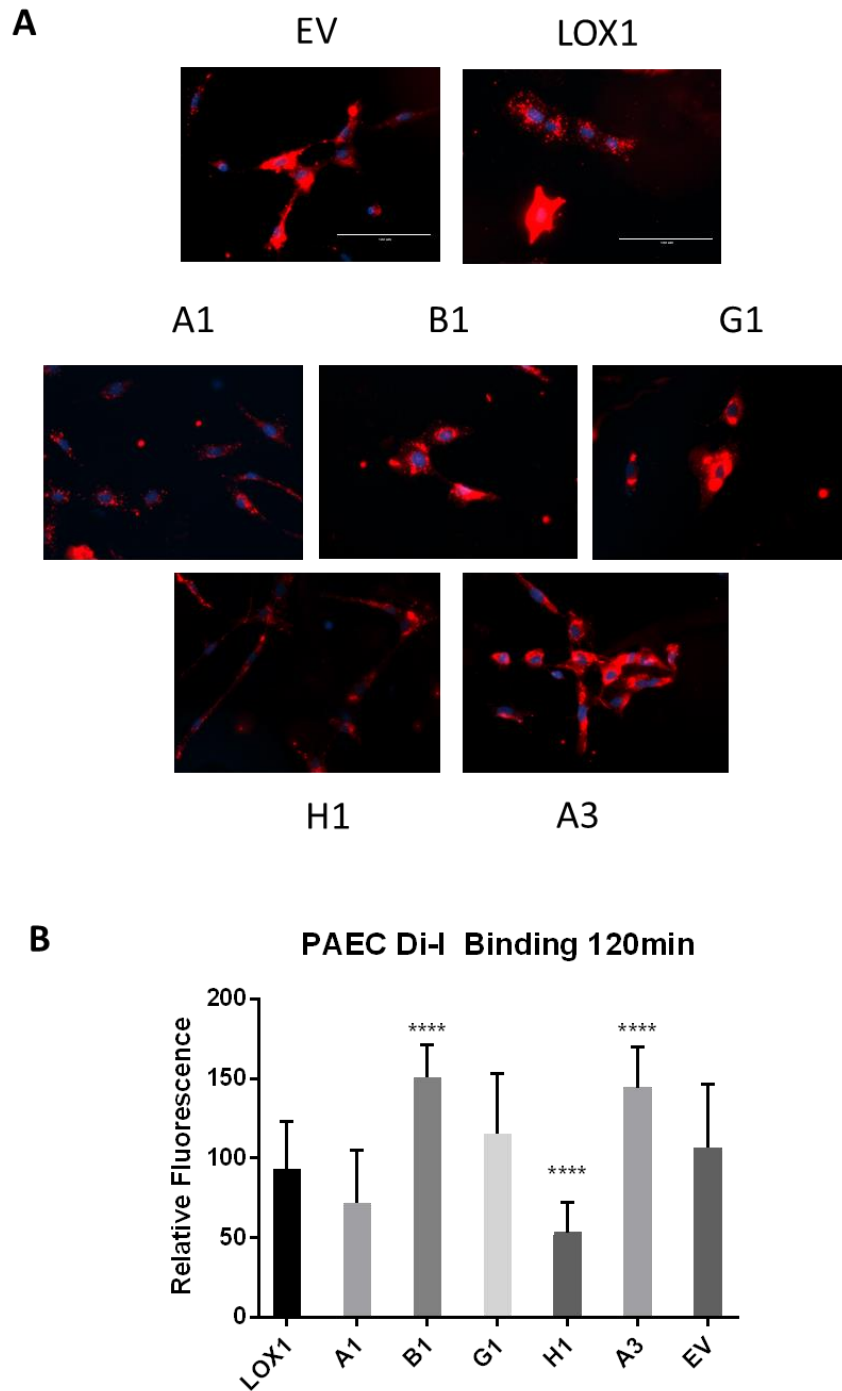
Legend: A) Binding of Di-I-oxLDL particles to negative control (EV) or LOX-1-expressing cells in the presence of Affimer (5 ug/ml). Nuclear stain, DAPI (blue); Di-I-oxLDL (red). Scale bar denotes 100micrometres (B) Quantification of Di-I-oxLDL binding in positive control LOX-1-expressing PAECs vs. Affimer-treated LOX-1-expressing PAECs. Error bars indicate +SEM; ****, p<0.0001

4.2.8   Affimer toxicity assay

A cell viability study was performed to assess the toxicity of Affimer at the concentrations used in this study (as described in the Materials and Methods). The exposure of HEK-293T cell lines to tetracycline and subsequent expression of LOX-1 was associated with a minor absolute decrease in cell viability (100% to 95%) after 24 hours. Incubation with Affimers for 24 hours did not bring about any change in cell viability. 2-way ANOVA did not demonstrate any statistically significant difference between groups (Figure 4-10A).

The PAEC cell lines were similar in response, though seemed to demonstrate that viability increased following tetracycline-dependent LOX-1-FLAG protein expression (76% to 80%). Co-incubation with affimers did not negatively affect cell viability. No results were statistically significant as assessed by 2-way ANOVA (Figure 4-10B).

Figure 4-10: Cell Viability Assays
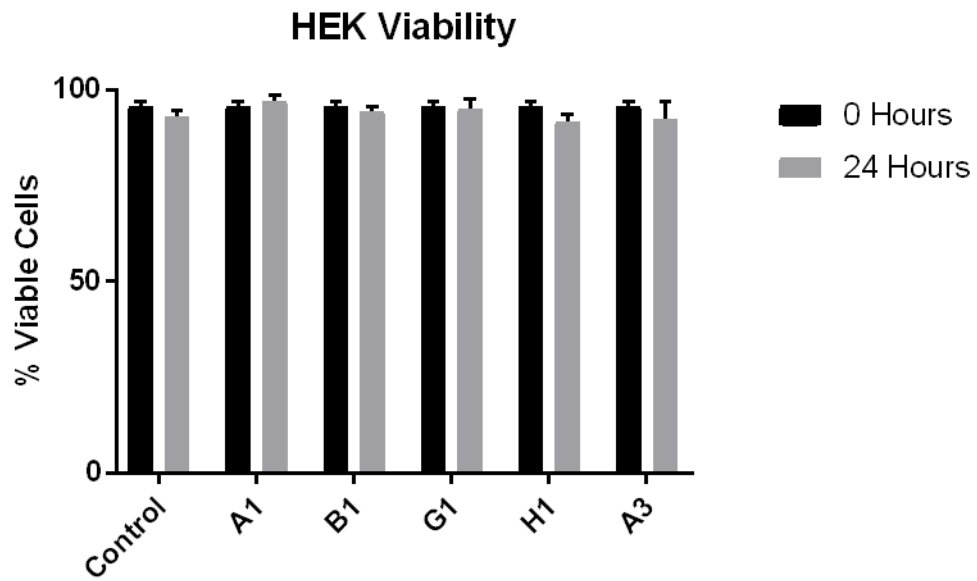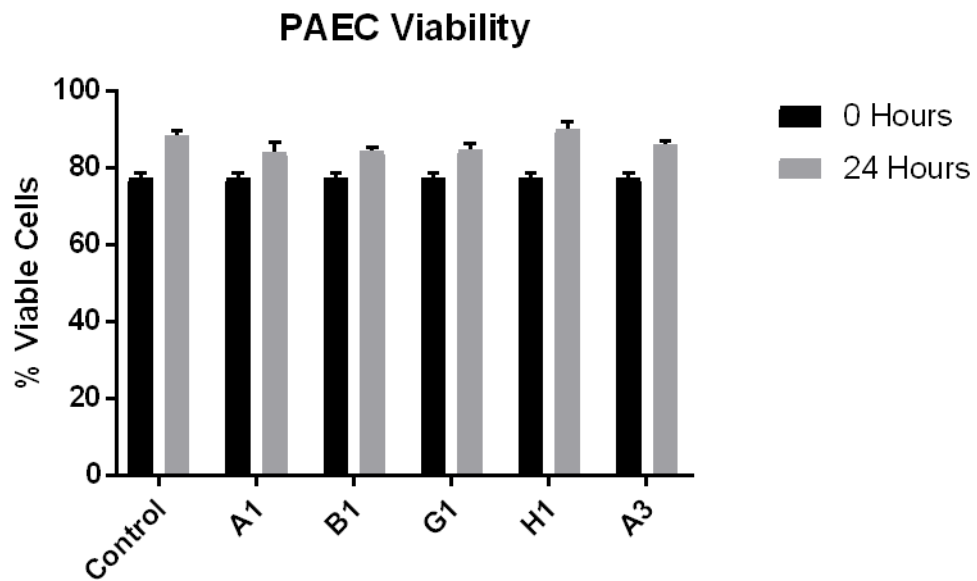
Legend: (A) Cell Viability assay of HEK293T cells after co-incubation with Afimers over 24 hours after induction of LOX-1 Flag expression. No statistically significant difference when compared to cells not exposed to Affimers. (B) Cell Viability assay of PAECs after co-incubation with Afimers over 24 hours after induction of LOX-1 Flag expression. No statistically significant difference when compared to cells not exposed to Affimers.

4.2.9   Flow Cytometry of HEK293T Cells and PAECs

Flow cytometry was undertaken as a more robust method to assess LOX-1 expression and cell viability. This is especially relevant as LOX-1 may promote pro-apoptotic signal transduction and cell death in response to oxLDL binding. In both HEK-293T and PAEC cell lines, after appropriate gating, histograms for EV and LOX-1-transfected cells looked very similar, with no visible shift favouring either greater or lesser fluorescence in response to excitation at the appropriate (560 nm) wavelength (Fig. 4.10). In line with this, pre-incubation with Affimers led to no difference in histogram shapes, compared to LOX-1-transfected positive controls (Fig. 4.11). Overall, signal levels were very low and cellular debris accounted for a significant proportion of events (45-65%). It was therefore unclear whether cells or fluorescence had degraded in the lead time between experiments, fixation and flow cytometry (approximately 24 hours).

Figure 4-11: Flow Cytometry

Population	Events	% Total	Median
EV	5149	51	8
LOX-1	5394	62	6
A1	4639	61	7
B1	5322	60	8
G1	5016	59	7
H1	3237	64	5
A3	3649	53	5

Population	Events	% Total	Median
EV	2699	27	10
LOX-1	1846	18	6
A1	1933	19	6
B1	2564	26	6
G1	2313	23	6
H1	2190	22	6
A3	2127	21	7
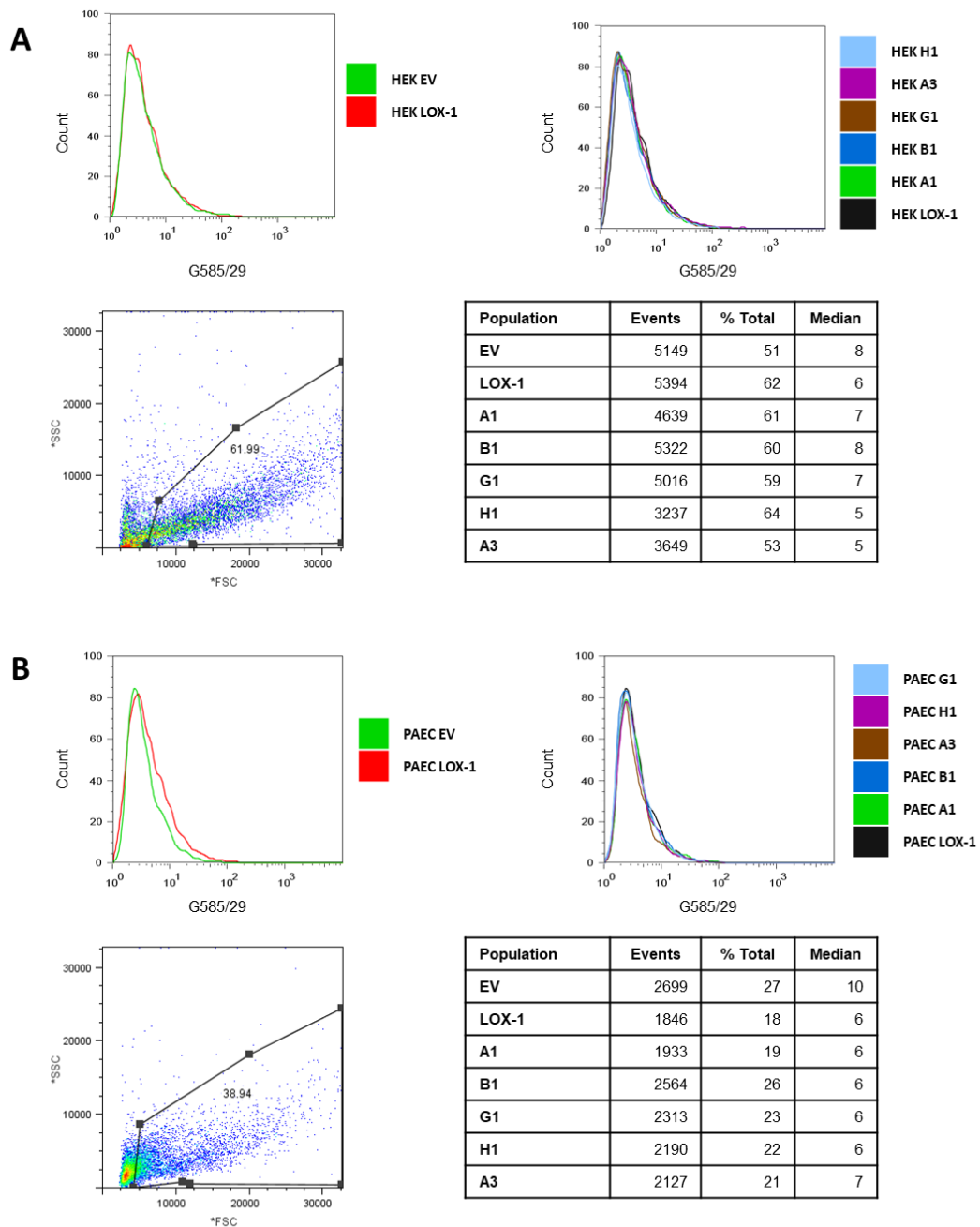
Legend: A) HEK 293 cells demonstrate no histogram shift between LOX-1 and EV after incubation with di-I oxLDL for 180 minutes. Affimers also do not promote any change. B) PAECs also demonstrate no histogram shift between LOX-1 and EV after incubation with di-I oxLDL for 120 minutes and Affimers have no effect. Both cell populations demonstrate a large amount of cell debris.

4.3 Discussion

This chapter aimed to establish whether Affimers directed against LOX-1 could disrupt the receptor function at the cell surface. To do this, two cell lines were employed. Firstly, a human kidney embryonic cell not known to natively express LOX-1 and secondly, an immortalised porcine vascular endothelial cell, which did not express human LOX-1. Both of these cell lines had been transfected with an inducible LOX-1 expression system.

Both cell lines were chosen as the presence or absence of human LOX-1 at the cell surface could be regulated and thus, EV-transfected cells could be used as a reliable negative control. The choice of cell lines may be criticised insofar as it did not include primary human endothelial cell lines, and indeed, as this work is continued, these should be the next cells studied. However, primary human cell lines present a challenge in their limited passage number (suppliers advise no more than 10 passages), relatively high cost and their plurality of scavenger receptors which bind modified forms of LDL (Abdul Zani et al., 2015). They were therefore not deemed suitable as an initial cell line with which to study the effects of Affimers.

The tetracycline-dependent, inducible LOX-1 system proved reliable in both cell lines. Using tubulin as a Western blotting control, the relative expression of human LOX-1 was significantly higher than in cells transfected with EV. This was supported by immunofluorescence microscopy which demonstrated clear fluorescence in these cell lines, in response to immunostaining with FLAG antibodies. Furthermore, the expression of LOX-1 was not associated with a drop in overall cell viability, suggesting

that protein expression and exposure to tetracycline was not inherently deleterious to the cell, a concern which has been raised in recent studies (Moullan et al., 2015).

The effectiveness of inducible Human LOX-1 expression in relation to the early binding of oxLDL could be clearly appreciated in HEK293Ts. This is an expected effect as ordinarily, scavenger receptor expression is increased hours after exposure to modified LDL (Martin-Fuentes et al., 2007). Thus, the pre-expressed receptors at the surface of cells are bound to have notable early effects. Supporting the hypothesis presented in this thesis, pre-incubation with four different Affimers significantly inhibited the binding of oxLDL at the cell surface. Moreover, this followed a dose-dependent effect, which seemed clearest with Affimers A1 and H1.

PAEC results broadly mirrored those of HEK293Ts: pre-expression with LOX-1 led to more oxLDL binding compared to EV controls. However, in contrast to HEK293Ts, PAECs seemed to natively bind oxLDL more readily, in the short incubation time-frame. The reason for this is not immediately clear but it may be that the baseline expression of scavenger receptors is higher in endothelial cell lines. Alternatively, the two hours of starvation preceding the experiment, designed to bring about cell quiescence, may have also brought about a stress response from cells, leading to upregulation of scavenger receptors (Abdul Zani et al., 2015).

When subjected to longer incubation, the effects of both the LOX-1 expression system and Affimers were less evident in HEK 293Ts. The overall difference in oxLDL binding between LOX-1 cells and EV was much less pronounced across. Thus, the spectrum by which Affimers could bring about change was much narrower and indeed, the effects were subdued. It was postulated that these effects may be unique

to the embryonic cell line. However, in PAECs, the effects of prolonged incubation were again, less pronounced and the effects of Affimers limited.

Given the limited sampling available through digital imaging software analysis (dozens of cells) and inherent heterogeneity in image selection, flow cytometry was employed as it allowed the sampling of a greater number of cells (thousands). However, this showed no discernible difference between cell lines or Affimer effects.

In summary, the pre-expression of LOX-1 leads to increased early ox-LDL binding, in both cell lines studied. In support of the working hypothesis, this effect is mitigated by Affimers. After prolonged incubation, negative controls bind oxLDL more readily and the early benefit of Affimers is nulled. It is presumed that this 'catch-up' effect is brought about by the expression of alternative scavenger receptors, oxLDL receptors or porcine LOX-1 receptors, to which Affimers do not bind. HEK293T cells are not known to express LOX-1, CD68, CD36 (Xu, S. et al., 2013) or SRA natively (Shannahan et al., 2015). Therefore, the binding of oxLDL must be brought about by an alternative receptor in this cell line. Regardless of which pathway is activated for the uptake of oxLDL, Affimers did not serve to inhibit this in either cell line. However, LOX-1 has been identified as the major oxLDL receptor in human endothelial cells and Affimers directed to human LOX-1 may bring about a more pronounced effect in human arterial endothelial cell lines. Future work should focus on primary cells and aim to translate the above findings to more clinically relevant endpoints through translational research.

CHAPTER 5 : DISCUSSION

5.1   General Discussion

Ischaemic heart disease and stroke are two of the three leading causes of death for men and women in England (Public Health England, 2017). The pathophysiology of ischaemic heart disease and stroke is multifactorial (Marzilli et al., 2012; Deb et al., 2010), however, atherosclerosis plays a fundamental part in their development (Marulanda-Londoño and Chaturvedi, 2016; Hansson, 2005). Further, non-fatal atherosclerosis carries significant associated morbidity, particularly when taken in context of its additional manifestations, such as peripheral arterial disease (Malyar et al., 2013).

Atherosclerosis is a chronic inflammatory process which is discussed more fully in the introduction, however, at its earliest stage can be seen as the deposition of lipids in the arterial subendothelium, through endothelial dysfunction (Hadi et al., 2005). Macrophages, smooth muscle cells, platelets and endothelial cells themselves react to such lipids and the paracrine effects brought about by the endothelial dysfunction to propagate further inflammation and atherosclerosis. LOX-1 is a scavenger receptor found in the cell walls of the above cells. Its over-expression is associated with increased atherosclerosis (Akhmedov et al., 2014). A number of cellular and translational studies have demonstrated that the suppression of LOX-1 is associated with a reduction in intra-arterial lipid deposition, and organ dysfunction (De Siqueira et al., 2015). Additionally, increases in the serum concentration of soluble-LOX-1 fragments are associated with negative outcomes in atherosclerotic disease (Pirillo

and Catapano, 2013). LOX-1 is therefore considered a potential target for preventing atherosclerosis and its clinically relevant sequelae (Mehta, Jawahar L. et al., 2011).

There are, however, key limitations in the technology currently available for the inhibition of LOX-1. Namely, that gene therapy and autoantibody treatment carry significant cost and unknown toxicity and its use for the prevention of disease is therefore unjustified. Therefore, in order the advance potential therapies, new technologies need to be developed. To date, there are very few studies assessing the application of antibody mimetics targeted against LOX-1. One study has described the use of LOX-1-targeted nanobodies for the imaging of atherosclerotic plaques by PET-CT. However, such nanobodies showed poor uptake in atherosclerotic plaque-rich regions (de Vos et al., 2012), thus bringing into question their value over more-established, antibody-based labels. A second study (Thakkar et al., 2015) used virtual molecular screening techniques to identify five molecules which bind at the LOX-1/oxLDL binding site, of these, two molecules demonstrated functional inhibition of oxLDL uptake. This study in particularly interesting in so far as it did not use established antibody mimetic platforms but commercially available organic chemicals.

A further challenge in studying LOX-1 is that anecdotally, members of the Ponnambalam laboratory have found commercially available antibodies very unreliable. This has also been a point of discussion with members of other laboratories at conferences. Moreover, there is no consensus on the normal serum concentration of LOX-1, being 64pg/ml (Kobayashi et al., 2011) in one paper, <0.5ng/ml (Kume, N. et al., 2010) in another and 1.6mcg/ml (Inoue et al., 2010) in a

third . Of note, some of these ranges fall outside most commercially available ELISA Kits for LOX-1, which have a maximum working range of less than 5ng/ml, and authors have described using their own kits. While it is entirely feasible that different assays may have subtle discrepancies in their results, variations greater than three orders of magnitude are substantial and bring into question the reliability of antibody-based assays.

This thesis describes the use of specific antibody mimetics, Affimers, to two different applications, namely in the sensing of LOX-1 and in inhibiting its function as an oxLDL receptor. The success and limitations of these are discussed in detail below

5.2 Affimers as sensing molecules

The first working hypothesis, as stated in the thesis' aims was that an Affimer-based assay could be used to differentiate between normal and elevated levels of soluble LOX-1. To test this hypothesis, five pre-screened Affimers were used to construct variations on an immunoassay including chemiluminescence and antibody-Affimer hybrid constructs. After optimisation, the assay was successful in detecting recombinant, extra-cellular domain LOX-1 in concentration of 100ng/ml to 10mcg/ml, proving that an Affimer-based platform can be used in the construction of immunoassays.

While the work for this thesis was being undertaken, two key papers were published by other groups. The first paper was published at the very outset of the research period. It described the use of Affimers as a platform for a sandwich ligand binding assay of pigment epithelium-derived factor (PEDF), however, PEDF was only measured within a narrow concentration range of 0, 1 and 5 μg/ml (Straw et al.,

2013). The assay was therefore underdeveloped and served only as a simple positive/negative test. Furthermore, the application of such a technology is dependent on its ability to detect physiological levels of target and most disease biomarkers exist in concentrations outside this range. The use of chemiluminescence or hybrid platforms had not been previously described in an Affimer-based assay and this was a novel area of exploration. It was demonstrated that CLAAT mimics chemiluminescence enzyme immunoassay (CLEIA), a simple modification of colorimetric ELISA, designed to increase sensitivity. The Hybrid antibody/Affimer CLAAT was therefore a more sophisticated assay with a demonstrably wider, linear working range.

The second key paper to be published while work for this thesis was conducted, describes a hybrid, antibody/Affimer, chemiluminescence platform to detect Glypican-3, an emerging hepatocellular carcinoma tumour marker (Xie et al., 2017). In contrast to the assay developed in this thesis, the GPC3 assay had a detection range of 0.03ng/ml to 600ng/ml. Further, this group found that their assay correlated better with immunohistochemistry than other available antibody-based assays. Key to this group's work was the fact that the working range of their assay covered normal and pathological concentrations of their target biomarker. However, as stated above, physiological concentrations of LOX-1 have been reported as low as four orders of magnitude below the detection limit of the assay developed herein (Brinkley et al., 2008).

Naturally, the sensitivity of such an assay is dependent on a number of factors, chief of which is the binding affinity between an Affimer and its target. However, the

binding affinity of Affimers has been shown to be comparable to immunoglobulins (Tiede et al., 2014); though this will vary in accordance to each Affimer-target pair, as illustrated by the discussion above.

The Affimer-based assay was to be used for quantifying sLOX-1 in human blood. Despite efforts at further optimisation, the lower limit of detection did not equal the lowest sLOX-1 levels described in the literature. Given that the working range of the assay was below that reported in commercial kits, no attempt was made to compare it directly against another assay, and no comment can be made on its superiority or inferiority to established methods.

Sandwich immunoassays in particular are known to be limited in the detection of small proteins due to the proximity of binding sites (steric hinderance). At this stage, there were two available avenues, either the assay could be taken forward into testing banked human samples (in the hope that the actual range would fall within the sensitivity established to date), or an attempt could be made to develop an alternative platform with which to develop the Affimer assay. Given that the banked samples were a limited resource it was decided that the next step should be the development of a biosensor.

The biosensor platform chosen for further experiments was electrochemical impedance spectroscopy, a technique which allows analysis of the resistive and capacitive properties of an electrical circuit, based on the application of a small sinusoidal current at varying frequency. Specifically, biosensors can monitor the progress of a biological reaction happening at the electrode surface and there are a wealth of examples of enzymes, antibodies and nanoparticles used in the

construction of biosensors (Goode et al., 2016; Guan et al., 2004). The biosensor reaction electrode was constructed using DropSens gold electrodes and a novel, patented polymerisation protocol developed by Elisha Biosystems using octopamine and tyramine (structurally similar amines) as monomers. While the particular protocol for the binding of tyramine and octopamine to Affimers and inducing polymerisation is novel, the application of polytyramine as a binding layer for electrical biosensors is a well-established process (Miao et al., 2005; Situmorang et al., 1998). On reflection, the presence of Affimers on the tethering layer had not been assured. As mentioned in chapter 4's discussion, their behaviour in response to cyclic voltammetry is unknown and it is possible that they may have been denatured or layed down in a disorganised manner as a result of polymerisation of octopamine and tyramine monomers. Equally, cysteine-containing Affimers have a strong tendency to form dimers due to their terminal thiol groups. Given that they were successfully biotinylated for immunoassay, it is reasonable to assume that dimerization did not impede thiol-amine coupling to biosensor monomers, though it is a worthwhile consideration, nonetheless. Ultimately, the biosensor itself failed to detect variations in the concentrations of Affimers. In particular, the Nyquist plots generated through the biosensor were very unstable and data could only be used across limited concentration and frequency ranges. At these ranges, there was no discernible difference in signal.

A recently published study (Zhurauski et al., 2018) reports on the use of a capacitive Affimer biosensor for the detection of receptor tyrosine-protein kinase erbB-4. In contrast to the technique employed in this report, their group used interdigitated, electron-beam evaporator coated electrodes and Affimers were applied using self-

assembling monolayer chemistry, directly onto electrodes. The authors report that the biosensor has a limit of detection of less than 1pM. Evidently, the use of vapor coated electrodes and a different coating technique is possibly accountable for the difference in results. However, the techniques employed within this report are established (Taleat et al., 2014; Goode et al., 2016) and with more time, alternative binding techniques, such as that developed by Zhurauski may have led to a clinically practicable biosensor and it is possible that such a technology could be used to assess blood samples.

5.3   Affimers as inhibitors

The second working hypothesis of this thesis was that Affimers could inhibit the binding of oxLDL by LOX-1. To investigate this, two cell lines were used: one which is not known to express oxLDL binding receptors and a vascular endothelial cell line which does. The intended purpose of the first approach was that HEK cells would allow the study of the LOX-1 receptor in isolation and any oxLDL bound to the cell would be as a direct result of this. Further, the effect of Affimers could be directly linked to their potency in inhibiting the LOX-1 receptor. In support of this, the empty vector cell line had almost no red spectrum fluorescence after short term incubation, while the LOX-1 expressing cell line had notable red spectrum fluorescence. Furthermore, four of the five Affimers brought about a significant reduction in the fluorescence of the LOX-1 cell lines, and by inference, inhibited the uptake of oxLDL. The fifth Affimer (G1) seemed to enhance oxLDL binding over and above that of LOX-1 transfected cells. This was evident across a range of concentration and suggested that G1 may bind allosterically and promote some conformational change in LOX-1

to boost its binding ability. However, such change was not evident on prolonged incubation or in experiments performed on PAECs, so may have been an erroneous finding. Short term incubation of oxLDL with PAECs revealed very similar results to those of HEK293s: all Affimers brought about a significant reduction in the binding of oxLDL. It was notable that there seemed to be a higher red spectrum fluorescence of the EV cell line; as discussed earlier, this may be due to the presence of natively expressed SRs in the PAEC cell line.

Two recently published studies have examined the role of Affimers as tools in inhibiting cellular function. The first, (Robinson et al., 2017) demonstrated that Affimers can block immunoglobulins from binding Fc gamma receptors in monocytes. A further study, took a very different approach by transfecting HEK293 cells with an anti-SUMO Affimer DNA sequence (Hughes et al., 2017). The study concluded that such Affimers were able to inhibit intracellular SUMO interaction motif-dependent protein-protein interactions. Lastly, though not a cellular function experiment, one group (King et al., 2015) have shown that Affimers can modulate C3 induced thrombolysis in fibrin clots, though published as an abstract, this work does not seem to have been taken further.

Unfortunately, when extended to a prolonged incubation time (2-3 hours). Immunofluorescence microscopy and subsequent image analysis demonstrated that the inducible LOX-1 expression system showed only a minor increase in the uptake of oxLDL, presumably due to other mechanisms within the cells studied being upregulated and 'catching up' with oxLDL binding and uptake. Thus, the effects of Affimers were not as evident, as might be expected, given that they bind human LOX-

1 and this is not natively expressed in either cell line. While there were small but statistically significant differences in the IF experiments, there was no discernible difference when cells were analysed by flow cytometry. Evidently, to investigate whether Affimers have a sustained effect on the LOX-1 receptor, it would be necessary to use them with a cell line which lacks other scavenger receptors, or oxLDL binding proteins. However, this was the intended purpose of using HEK293 cells. An alternative approach would be to use a cell line which is known to express relevant receptors but to knock them out genetically, though this would be highly impractical and translation directly into primary human endothelial cells is a more natural next step,

Only one published study has shown a sustained effect of Affimers on cell function: Affimers targeted against vascular endothelial growth factor (VEGF) receptor bring about a reduction in tubulogenesis in human umbilical vein endothelial cells in response to VEGF stimulation(Tiede et al., 2017). Whether a similar effect could be demonstrated on the binding of oxLDL to LOX-1 remains unclear. However, it has been demonstrated that LOX-1 continually cycles between endosomes and the cell membrane leading to dissociation from LOX-1 bound oxLDL after approximately 30 minutes (Murphy et al., 2008). Attempts were made to study the co-localisation of oxLDL and LOX-1 in the experimental models of this thesis, however, the use of membrane solubilisers which would allow antibody probing for LOX-1 seemed to solubilise oxLDL and this experiment was not possible. Nonetheless, this poses an interesting question as to whether Affimers, bound to LOX-1 would be internalised and lysed by intra-cellular mechanisms, or whether they would bind to LOX-1 in an irreversible manner, hence bringing about a sustained effect.

Lastly, the effects of the LOX-1 expression system and Affimers on cell viability warrant discussion. As reflected in the results, exposure of the cell to both of these brought about no significant decrease in cell viability over 24 to 36 hours. It is possible, nonetheless, that the cell is stressed by either the over-expression of LOX-1 or by any undiscovered toxicity brought about by Affimers. No attempt has been made within this thesis to study whether pro-apoptotic signalling pathways are upregulated in this thesis. Such work was partly carried out in parallel by another member of the Ponnambalam laboratory (Abdul Zani, 2016), who found that the inducible expression of LOX-1 brought about an increase in pro-inflammatory and pro-apoptotic signalling. The effects of Affimers on these pathways remains elusive.

5.4   Future Work

While successful in achieving some its aims, this thesis leaves some questions unanswered. Firstly, although an Affimer-based serum LOX-1 affinity assay or biosensor which works within physiological limits appears within reach, it remains unclear what long-term use such a tool will provide. Examining successfully developed sensors above, the path to develop a LOX-1 biosensor seems clear. Future questions to be answered for when such a sensor is developed will revolve around whether LOX-1 elevation correlates with increased long-term risk of atherosclerotic disease or metabolic syndrome, as had been hoped at the commencement of this work. Although work carried out in a Japanese population (Inoue et al., 2010) would suggest not, European populations are subjected to very different diet and genetics. Further, LOX-1 is rapidly elevated in myocardial ischaemia, a time-critical, acute condition. A LOX-1 antibody ELISA takes hours to conduct and is very user dependent.

By contrast, a calibrated biosensor can generate results in minutes, making the rapid assessment and treatment of myocardial infarcts more viable.

The use of Affimers as LOX-1 inhibitors carries much greater potential. Affimers are one of many molecules which make up the expanding field of nanobody particle (or antibody mimetic) research. As discussed in the introduction, despite their enormous potential, nanobodies have had limited success in their practical application. This is perhaps best understood when reviewing the history of aptamers, the oldest and best established nanobody molecules (Ellington and Szostak, 1990). Though they have been successfully used as sensing molecules (Feng et al., 2011) and viral inhibitors (Binning et al., 2012), their translation into clinical research has been limited by rapid degradation, renal excretion, undefined pharmacokinetics, and molecule cross-reactivity (Lakhin et al., 2013). These challenges remain untested for Affimers and their future development into viable therapeutic agents will depend on their success.

The obvious next step for the development of Affimers as a therapeutic agent is to establish their effect on a primary human vascular endothelial cell line. Such work should focus on short term and medium-term effect on oxLDL binding and the localisation of Affimers and LOX-1. While no Affimer-specific immunofluorescent labels are available currently, a protein tag could easily be engineered into the plasmid vector for Affimers, as has already been described (Lopata et al., 2018). Should Affimers demonstrate inhibition of oxLDL binding *in vitro*, the next step should be their application in an *in vivo* model. The Ponnambalam laboratory keeps LOX-1 and ApoE knockout mice and these would be excellent experimental and control

subjects for future experiments. Naturally, human LOX-1 Affimers will need to be screened against the murine equivalent to assess cross reactivity, if none is established, a new round of screening will need to be performed. A reproduction of prior research findings such as reduced atherosclerosis and reduction of myocardial infarct areas would be key areas to examine.

An obvious limitation of the use of Affimers as therapeutic agents lie in their small size. Though a desirable characteristic for tissue and cellular penetration, this leaves them vulnerable to renal clearance once used systemically. Such challenges may be overcome by binding the Affimers to larger, inert molecules such as polyethylene glycol (Veronese and Mero, 2008). Linked to this will be the pharmacokinetics of LOX-1-targeted Affimers, the ideal dose and dosing schedule. However, this will not become apparent until animal-based research begins. Regardless, concerns will remain about the immunogenic potential of Affimers. While the immunogenic potential of Affimers have been publicised as very low by Avacta (Avacta Life Sciences, 2017), a private pharmaceutical company currently licensing the technology, such data has not been published in a peer-reviewed publication (though this is not customary).

5.5 Closing Remarks

While the work in this thesis has predominantly focused on the development of Affimer based LOX-1 assays and inhibition, it has only touched lightly on the use of alternative technologies to achieve the same goal and in the use of the same technology to achieve alternative goals. Examples of this include the use of Affimers for near molecular-level microscopy labelling (Schlichthaerle et al., 2018) (a

technique not possible with the use of antibody-based techniques, due to their large size) and the recent discovery of LOX-1 binding small molecules not based on existing nanobody structures (Thakkar et al., 2015).

Should viable Affimer-based, LOX-1-modulating therapies be developed, key questions will remain as to what their role should be. Given their protein-based structure, they are unlikely to be delivered orally, unless significant advances are made in oral peptide administration (Gupta et al., 2013). Thus, a significant therapeutic benefit will need to be proven to justify the frequent administration of intravenous Affimers. Such benefit is unlikely to be proven in the prevention of atherosclerotic disease, a chronic condition manifesting over decades and subject to significant modulation by current prevention strategies. Nonetheless, as discussed in the introduction, LOX-1 expression is also elevated in the presence of other inflammatory conditions such as arthritis (Hashimoto et al., 2017), pre-eclampsia (Chigusa et al., 2012) and sepsis (Wu et al., 2011). Furthermore, the administration of anti-LOX-1 antibodies has led to the abrogation of some of these inflammatory conditions in animal models(Ishikawa et al., 2012; Landsberger et al., 2010). It is likely that the parenteral administration of LOX-1 Affimers would be more easily justified, in such conditions which are immediately life-threatening or debilitating. These conditions have not formed the focus of this thesis, but the progress made herein may lead to applicable benefits. There is clearly great scope for further research in this field and future developments are eagerly anticipated.

REFERENCES

Abdul Zani, I. 2016. *P46 Oxidised LDL and LOX-1 scavenger receptor regulation of pro-atherogenic signal transduction (PhD Thesis, University of Leeds).*

Abdul Zani, I., Stephen, S.L., Mughal, N.A., Russell, D., Homer-Vanniasinkam, S., Wheatcroft, S.B. and Ponnambalam, S. 2015. Scavenger Receptor Structure and Function in Health and Disease. *Cells.* **4**(2), pp.178-201.

Adachi, H., Tsujimoto, M., Arai, H. and Inoue, K. 1997. Expression cloning of a novel scavenger receptor from human endothelial cells. *J Biol Chem.* **272**(50), pp.31217-31220.

Akamatsu, T., Dai, H., Mizuguchi, M., Goto, Y., Oka, A. and Itoh, M. 2014. LOX-1 is a novel therapeutic target in neonatal hypoxic-ischemic encephalopathy. *Am J Pathol.* **184**(6), pp.1843-1852.

Akhmedov, A., Rozenberg, I., Paneni, F., Camici, G.G., Shi, Y., Doerries, C., Sledzinska, A., Mocharla, P., Breitenstein, A., Lohmann, C., Stein, S., von Lukowicz, T., Kurrer, M.O., Boren, J., Becher, B., Tanner, F.C., Landmesser, U., Matter, C.M. and Luscher, T.F. 2014. Endothelial overexpression of LOX-1 increases plaque formation and promotes atherosclerosis in vivo. *Eur Heart J.* **35**(40), pp.2839-2848.

Allen, J.E., Ferrini, R., Dicker, D.T., Batzer, G., Chen, E., Oltean, D.I., Lin, B., Renshaw, M.W., Kretz-Rommel, A. and El-Deiry, W.S. 2012. Targeting TRAIL death receptor 4 with trivalent DR4 Atrimer complexes. *Mol Cancer Ther.* **11**(10), pp.2087-2095.

Amine, A., Mohammadi, H., Bourais, I. and Palleschi, G. 2006. Enzyme inhibition-based biosensors for food safety and environmental monitoring. *Biosens Bioelectron.* **21**(8), pp.1405-1423.

Anderson, G.P., Hohlbaum, A., Jensen, K., Bähre, A. and Gille, H. 2015. Discovery of PRS-060, an inhalable CD123/IL4Ra/TH2 blocking anti-asthmatic anticalin protein re-engineered from endogenous lipocalin-1. *European Respiratory Journal.* **46**(suppl 59).

Aoyama, T., Sawamura, T., Furutani, Y., Matsuoka, R., Yoshida, M.C., Fujiwara, H. and Masaka, T. 1999. Structure and chromosomal assignment of the human lectin-like oxidized low-density-lipoprotein receptor-1 (LOX-1) gene. *Biochemical Journal.* **339**(1), pp.177-184.

Arbogast, B.W., Lee, G.M. and Raymond, T.L. 1982. In Vitro Injury of Porcine Aortic Endothelial Cells by Very-Low-Density Lipoproteins from Diabetic Rat Serum. *Diabetes.* **31**(7), pp.593-599.

Avacta Life Sciences. 2017. *No immunogenicity issues for Affimer technology*. [Press release]. [Accessed 27/04/2019]. Available from: https://www.avacta.com/blogs/no-immunogenicity-issues-affimer-technology

Aydemir, N., Malmstrom, J. and Travas-Sejdic, J. 2016. Conducting polymer based electrochemical biosensors. *Physical Chemistry Chemical Physics.* **18**(12), pp.8264-8277.

Bahadir, E.B. and Sezginturk, M.K. 2016. A review on impedimetric biosensors. *Artif Cells Nanomed Biotechnol.* **44**(1), pp.248-262.

Basini, G., Santini, S.E., Bussolati, S. and Grasselli, F. 2007. Sanguinarine inhibits VEGF-induced Akt phosphorylation. *Ann N Y Acad Sci.* **1095**, pp.371-376.

Beckman, R.A., Weiner, L.M. and Davis, H.M. 2007. Antibody constructs in cancer therapy: protein engineering strategies to improve exposure in solid tumors. *Cancer.* **109**(2), pp.170-179.

Berenson , G.S., Srinivasan , S.R., Bao , W., Newman , W.P., Tracy , R.E. and Wattigney , W.A. 1998. Association between Multiple Cardiovascular Risk Factors and Atherosclerosis in Children and Young Adults. *New England Journal of Medicine.* **338**(23), pp.1650-1656.

Binning, J.M., Leung, D.W. and Amarasinghe, G.K. 2012. Aptamers in virology: recent advances and challenges. *Front Microbiol.* **3**, p29.

Biocca, S., Falconi, M., Filesi, I., Baldini, F., Vecchione, L., Mango, R., Romeo, F., Federici, G., Desideri, A. and Novelli, G. 2009. Functional Analysis and Molecular Dynamics Simulation of LOX-1 K167N Polymorphism Reveal Alteration of Receptor Activity. *PLoS ONE.* **4**(2), pe4648.

Bobryshev, Y.V. 2006. Monocyte recruitment and foam cell formation in atherosclerosis. *Micron.* **37**(3), pp.208-222.

Boccafoschi, F., Habermehl, J., Vesentini, S. and Mantovani, D. 2005. Biological performances of collagen-based scaffolds for vascular tissue engineering. *Biomaterials.* **26**(35), pp.7410-7417.

Bossaller, L. and Rothe, A. 2013. Monoclonal antibody treatments for rheumatoid arthritis. *Expert Opin Biol Ther.* **13**(9), pp.1257-1272.

Bradbury, A. and Pluckthun, A. 2015. Reproducibility: Standardize antibodies used in research. *Nature.* **518**(7537), pp.27-29.

Brinkley, T.E., Kume, N., Mitsuoka, H., Phares, D.A. and Hagberg, J.M. 2008. Elevated Soluble Lectin-like Oxidized LDL Receptor 1 (LOX-1) Levels in Obese Postmenopausal Women. *Obesity (Silver Spring, Md.).* **16**(6), pp.1454-1456.

Canton, J., Neculai, D. and Grinstein, S. 2013. Scavenger receptors in homeostasis and immunity. *Nat Rev Immunol.* **13**(9), pp.621-634.

Chambers, J.P., Arulanandam, B.P., Matta, L.L., Weis, A. and Valdes, J.J. 2008. Biosensor recognition elements. *Curr Issues Mol Biol.* **10**(1-2), pp.1-12.

Chames, P., Van Regenmortel, M., Weiss, E. and Baty, D. 2009. Therapeutic antibodies: successes, limitations and hopes for the future. *British Journal of Pharmacology.* **157**(2), pp.220-233.

Chang, B., Gray, P., Piltch, M., Bulgin, M.S., Sorensen-Melson, S., Miller, M.W., Davies, P., Brown, D.R., Coughlin, D.R. and Rubenstein, R. 2009. Surround optical fiber immunoassay (SOFIA): an ultra-sensitive assay for prion protein detection. *J Virol Methods.* **159**(1), pp.15-22.

Chapman, M.J., Goldstein, S., Lagrange, D. and Laplaud, P.M. 1981. A density gradient ultracentrifugal procedure for the isolation of the major lipoprotein classes from human serum. *J Lipid Res.* **22**(2), pp.339-358.

Chen, H., Li, D., Sawamura, T., Inoue, K. and Mehta, J.L. 2000. Upregulation of LOX-1 Expression in Aorta of Hypercholesterolemic Rabbits: Modulation by Losartan. *Biochemical and Biophysical Research Communications.* **276**(3), pp.1100-1104.

Chen, J., Mehta, J.L., Haider, N., Zhang, X., Narula, J. and Li, D. 2004. Role of Caspases in Ox-LDL–Induced Apoptotic Cascade in Human Coronary Artery Endothelial Cells. *Circulation Research.* **94**(3), pp.370-376.

Chen, M., Kakutani, M., Minami, M., Kataoka, H., Kume, N., Narumiya, S., Kita, T., Masaki, T. and Sawamura, T. 2000. Increased Expression of Lectinlike Oxidized Low Density Lipoprotein

Receptor-1 in Initial Atherosclerotic Lesions of Watanabe Heritable Hyperlipidemic Rabbits. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **20**(4), pp.1107-1115.

Chen, M., Kakutani, M., Naruko, T., Ueda, M., Narumiya, S., Masaki, T. and Sawamura, T. 2001. Activation-dependent surface expression of LOX-1 in human platelets. *Biochem Biophys Res Commun.* **282**(1), pp.153-158.

Chen, Q., Reis, S.E., Kammerer, C., Craig, W.Y., LaPierre, S.E., Zimmer, E.L., McNamara, D.M., Pauly, D.F., Sharaf, B., Holubkov, R., Bairey Merz, C.N., Sopko, G., Bontempo, F. and Kamboh, M.I. 2003. Genetic Variation in Lectin-Like Oxidized Low-Density Lipoprotein Receptor 1 (LOX1) Gene and the Risk of Coronary Artery Disease. *Circulation.* **107**(25), pp.3146-3151.

Chiba, Y., Ogita, T., Ando, K. and Fujita, T. 2001. PPARgamma ligands inhibit TNF-alpha-induced LOX-1 expression in cultured endothelial cells. *Biochem Biophys Res Commun.* **286**(3), pp.541-546.

Chigusa, Y., Tatsumi, K., Kondoh, E., Fujita, K., Nishimura, F., Mogami, H. and Konishi, I. 2012. Decreased lectin-like oxidized LDL receptor 1 (LOX-1) and low Nrf2 activation in placenta are involved in preeclampsia. *J Clin Endocrinol Metab.* **97**(10), pp.E1862-1870.

Chung, D.-J., Kim, K.-C. and Choi, S.-H. 2011. Electrochemical DNA biosensor based on avidin–biotin conjugation for influenza virus (type A) detection. *Applied Surface Science.* **257**(22), pp.9390-9396.

Cinti, S., Mazzaracchio, V., Cacciotti, I., Moscone, D. and Arduini, F. 2017. Carbon Black-Modified Electrodes Screen-Printed onto Paper Towel, Waxed Paper, and Parafilm M(®). *Sensors (Basel, Switzerland).* **17**(10), p2267.

Clark, L.C., Jr. and Lyons, C. 1962. Electrode systems for continuous monitoring in cardiovascular surgery. *Ann N Y Acad Sci.* **102**, pp.29-45.

Clark, L.C., Jr., Wolf, R., Granger, D. and Taylor, Z. 1953. Continuous recording of blood oxygen tensions by polarography. *J Appl Physiol.* **6**(3), pp.189-193.

Collaborators, G.M.a.C.o.D. 2015. Global, regional, and national age–sex specific all-cause and cause-specific mortality for 240 causes of death, 1990–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet.* **385**(9963), pp.117-171.

Collot-Teixeira, S., Martin, J., McDermott-Roe, C., Poston, R. and McGregor, J.L. 2007. CD36 and macrophages in atherosclerosis. *Cardiovasc Res.* **75**(3), pp.468-477.

Creager , M.A., Kaufman , J.A. and Conte , M.S. 2012. Acute Limb Ischemia. *New England Journal of Medicine.* **366**(23), pp.2198-2206.

Cushing, S.D., Berliner, J.A., Valente, A.J., Territo, M.C., Navab, M., Parhami, F., Gerrity, R., Schwartz, C.J. and Fogelman, A.M. 1990. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proceedings of the National Academy of Sciences.* **87**(13), pp.5134-5138.

Danesh, J., Lewington, S., Thompson, S.G., Lowe, G.D., Collins, R., Kostis, J.B., Wilson, A.C., Folsom, A.R., Wu, K., Benderly, M., Goldbourt, U., Willeit, J., Kiechl, S., Yarnell, J.W., Sweetnam, P.M., Elwood, P.C., Cushman, M., Psaty, B.M., Tracy, R.P., Tybjaerg-Hansen, A., Haverkate, F., de Maat, M.P., Fowkes, F.G., Lee, A.J., Smith, F.B., Salomaa, V., Harald, K., Rasi, R., Vahtera, E., Jousilahti, P., Pekkanen, J., D'Agostino, R., Kannel, W.B., Wilson, P.W., Tofler, G., Arocha-Pinango, C.L., Rodriguez-Larralde, A., Nagy, E., Mijares, M., Espinosa, R., Rodriquez-Roa, E., Ryder, E., Diez-Ewald, M.P., Campos, G., Fernandez, V., Torres, E., Marchioli, R., Valagussa, F., Rosengren, A., Wilhelmsen, L., Lappas, G., Eriksson, H., Cremer, P., Nagel, D., Curb, J.D., Rodriguez, B., Yano, K., Salonen, J.T., Nyyssonen, K., Tuomainen, T.P., Hedblad, B., Lind, P., Loewel, H., Koenig, W., Meade, T.W., Cooper, J.A., De Stavola, B., Knottenbelt, C., Miller, G.J., Cooper, J.A., Bauer, K.A., Rosenberg, R.D., Sato, S., Kitamura, A., Naito, Y., Palosuo, T., Ducimetiere, P., Amouyel, P., Arveiler, D., Evans, A.E., Ferrieres, J., Juhan-Vague, I., Bingham, A., Schulte, H., Assmann, G., Cantin, B., Lamarche, B., Despres, J.P., Dagenais, G.R., Tunstall-Pedoe, H., Woodward, M., Ben-Shlomo, Y., Davey Smith, G., Palmieri, V., Yeh, J.L., Rudnicka, A., Ridker, P., Rodeghiero, F., Tosetto, A., Shepherd, J., Ford, I., Robertson, M., Brunner, E., Shipley, M., Feskens, E.J., Kromhout, D., Dickinson, A., Ireland, B., Juzwishin, K., Kaptoge, S., Lewington, S., Memon, A., Sarwar, N., Walker, M., Wheeler, J., White, I. and Wood, A. 2005. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *Jama.* **294**(14), pp.1799-1809.

Danesh, J., Wheeler, J.G., Hirschfield, G.M., Eda, S., Eiriksdottir, G., Rumley, A., Lowe, G.D., Pepys, M.B. and Gudnason, V. 2004. C-reactive protein and other circulating markers of inflammation in the prediction of coronary heart disease. *N Engl J Med.* **350**(14), pp.1387-1397.

De Siqueira, J., Abdul Zani, I., Russell, D.A., Wheatcroft, S.B., Ponnambalam, S. and Homer-Vanniasinkam, S. 2015. Clinical and Preclinical Use of LOX-1-Specific Antibodies in Diagnostics and Therapeutics. *J Cardiovasc Transl Res.* **8**(8), pp.458-465.

de Vos, J., Broisat, A., Toczek, J., Ghezzi, C., Lahoutte, T., Muydermans, S. and Devoogdt, N. 2012. LOX-1 targeting Nanobodies for SPECT Imaging of Atherosclerosis. In: *Worlm Molecular Imaging Congress*, *Dublin*.

Deanfield, J.E., Halcox, J.P. and Rabelink, T.J. 2007. Endothelial Function and Dysfunction. *Testing and Clinical Relevance.* **115**(10), pp.1285-1295.

Deb, P., Sharma, S. and Hassan, K.M. 2010. Pathophysiologic mechanisms of acute ischemic stroke: An overview with emphasis on therapeutic significance beyond thrombolysis. *Pathophysiology.* **17**(3), pp.197-218.

Delneste, Y., Magistrelli, G., Gauchat, J., Haeuw, J., Aubry, J., Nakamura, K., Kawakami-Honda, N., Goetsch, L., Sawamura, T., Bonnefoy, J. and Jeannin, P. 2002. Involvement of LOX-1 in dendritic cell-mediated antigen cross-presentation. *Immunity.* **17**(3), pp.353-362.

Delost, M. 2014. Blood Gas and Critical Care Analyte Analysis. In: El-Khatib, T.A.V.R.L.C.M.F. ed. *Equipment for Respiratory Care.* Burlington, MA: Jones & Bartlett.

Desmet, J., Verstraete, K., Bloch, Y., Lorent, E., Wen, Y., Devreese, B., Vandenbroucke, K., Loverix, S., Hettmann, T., Deroo, S., Somers, K., Henderikx, P., Lasters, I. and Savvides, S.N. 2014. Structural basis of IL-23 antagonism by an Alphabody protein scaffold. *Nature Communications.* **5**, p5237.

Despres, J.P., Lemieux, I., Dagenais, G.R., Cantin, B. and Lamarche, B. 2000. HDL-cholesterol as a marker of coronary heart disease risk: the Quebec cardiovascular study. *Atherosclerosis.* **153**(2), pp.263-272.

Di Pietro, N., Formoso, G. and Pandolfi, A. 2016. Physiology and pathophysiology of oxLDL uptake by vascular wall cells in atherosclerosis. *Vascular Pharmacology.* **84**, pp.1-7.

Dominguez, J.H., Mehta, J.L., Li, D., Wu, P., Kelly, K.J., Packer, C.S., Temm, C., Goss, E., Cheng, L., Zhang, S., Patterson, C.E., Hawes, J.W. and Peterson, R. 2008. Anti-LOX-1 therapy in rats with diabetes and dyslipidemia: ablation of renal vascular and epithelial manifestations. *Am J Physiol Renal Physiol.* **294**(1), pp.F110-119.

Draggan, S. 1976. Improved specimen coating technique for scanning electron microscope observation of decomposer microorganisms. *Appl Environ Microbiol.* **31**(2), pp.313-315.

Ellington, A.D. and Szostak, J.W. 1990. In vitro selection of RNA molecules that bind specific ligands. *Nature.* **346**(6287), pp.818-822.

English, F.A., McCarthy, F.P., McSweeney, C.L., Quon, A.L., Morton, J.S., Sawamura, T., Davidge, S.T. and Kenny, L.C. 2013. Inhibition of lectin-like oxidized low-density lipoprotein-1 receptor protects against plasma-mediated vascular dysfunction associated with pre-eclampsia. *Am J Hypertens.* **26**(2), pp.279-286.

Engvall, E. and Perlmann, P. 1971. Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry.* **8**(9), pp.871-874.

Eto, H., Miyata, M., Kume, N., Minami, M., Itabe, H., Orihara, K., Hamasaki, S., Biro, S., Otsuji, Y., Kita, T. and Tei, C. 2006. Expression of lectin-like oxidized LDL receptor-1 in smooth muscle cells after vascular injury. *Biochem Biophys Res Commun.* **341**(2), pp.591-598.

Febbraio, M., Podrez, E.A., Smith, J.D., Hajjar, D.P., Hazen, S.L., Hoff, H.F., Sharma, K. and Silverstein, R.L. 2000. Targeted disruption of the class B scavenger receptor CD36 protects against atherosclerotic lesion development in mice. *J Clin Invest.* **105**(8), pp.1049-1056.

Feng, L., Chen, Y., Ren, J. and Qu, X. 2011. A graphene functionalized electrochemical aptasensor for selective label-free detection of cancer cells. *Biomaterials.* **32**(11), pp.2930-2937.

Fiedler, E., Fiedler, M., Proetzel, G., Scheuermann, T., Fiedler, U. and Rudolph, R. 2006. Affilin™ Molecules. *Food and Bioproducts Processing.* **84**(1), pp.3-8.

Firer, M.A. and Gellerman, G. 2012. Targeted drug delivery for cancer therapy: the other side of antibodies. *Journal of Hematology & Oncology.* **5**, pp.70-70.

Ford, E.S. and Caspersen, C.J. 2012. Sedentary behaviour and cardiovascular disease: a review of prospective studies. *International Journal of Epidemiology.* **41**(5), pp.1338-1353.

Frangogiannis, N.G. 2015. Pathophysiology of Myocardial Infarction. *Compr Physiol.* **5**(4), pp.1841-1875.

Galassi, A., Reynolds, K. and He, J. 2006. Metabolic syndrome and risk of cardiovascular disease: a meta-analysis. *Am J Med.* **119**(10), pp.812-819.

Gerhardt, T. and Ley, K. 2015. Monocyte trafficking across the vessel wall. *Cardiovasc Res.* **107**(3), pp.321-330.

Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S. 1979. Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proceedings of the National Academy of Sciences.* **76**(1), pp.333-337.

Gong, C., Chen, J., Song, Y., Sun, M., Song, Y., Guo, Q. and Wang, L. 2016. A glucose biosensor based on the polymerization of aniline induced by a bio-interphase of glucose oxidase and horseradish peroxidase. *Analytical Methods.* **8**(7), pp.1513-1519.

Goode, J., Dillon, G. and Millner, P.A. 2016. The development and optimisation of nanobody based electrochemical immunosensors for IgG. *Sensors and Actuators B: Chemical.* **234**, pp.478-484.

Grabulovski, D., Kaspar, M. and Neri, D. 2007. A novel, non-immunogenic Fyn SH3-derived binding protein with tumor vascular targeting properties. *J Biol Chem.* **282**(5), pp.3196-3204.

Gracy, J., Le-Nguyen, D., Gelly, J.-C., Kaas, Q., Heitz, A. and Chiche, L. 2008. KNOTTIN: the knottin or inhibitor cystine knot scaffold in 2007. *Nucleic Acids Research.* **36**(Database issue), pp.D314-D319.

Graham, A., Hogg, N., Kalyanaraman, B., O'Leary, V., Darley-Usmar, V. and Moncada, S. 1993. Peroxynitrite modification of low-density lipoprotein leads to recognition by the macrophage scavenger receptor. *FEBS Lett.* **330**(2), pp.181-185.

Grieshaber, D., MacKenzie, R., Vörös, J. and Reimhult, E. 2008. Electrochemical Biosensors - Sensor Principles and Architectures. *Sensors (Basel, Switzerland).* **8**(3), pp.1400-1458.

Grundy, S.M., Benjamin, I.J., Burke, G.L., Chait, A., Eckel, R.H., Howard, B.V., Mitch, W., Smith, S.C. and Sowers, J.R. 1999. Diabetes and Cardiovascular Disease. *A Statement for Healthcare Professionals From the American Heart Association.* **100**(10), pp.1134-1146.

Guan, J.-G., Miao, Y.-Q. and Zhang, Q.-J. 2004. Impedimetric biosensors. *Journal of Bioscience and Bioengineering.* **97**(4), pp.219-226.

Gupta, S., Jain, A., Chakraborty, M., Sahni, J.K., Ali, J. and Dang, S. 2013. Oral delivery of therapeutic proteins and peptides: a review on recent developments. *Drug Delivery.* **20**(6), pp.237-246.

Guy, R.A., Maguire, G.F., Crandall, I., Connelly, P.W. and Kain, K.C. 2001. Characterization of peroxynitrite-oxidized low density lipoprotein binding to human CD36. *Atherosclerosis.* **155**(1), pp.19-28.

Hadi, H.A.R., Carr, C.S. and Al Suwaidi, J. 2005. Endothelial Dysfunction: Cardiovascular Risk Factors, Therapy, and Outcome. *Vascular Health and Risk Management.* **1**(3), pp.183-198.

Hammami, A., Kuliček, J. and Raouafi, N. 2016. A naphthoquinone/SAM-mediated biosensor for olive oil polyphenol content. *Food Chemistry.* **209**, pp.274-278.

Hansson, G.K. 2005. Inflammation, Atherosclerosis, and Coronary Artery Disease. *New England Journal of Medicine.* **352**(16), pp.1685-1695.

Hashimoto, K., Oda, Y., Nakamura, F., Kakinoki, R. and Akagi, M. 2017. Lectin-like, oxidized low-density lipoprotein receptor-1-deficient mice show resistance to age-related knee osteoarthritis. *European Journal of Histochemistry : EJH.* **61**(1), p2762.

Hayashida, K., Kume, N., Murase, T., Minami, M., Nakagawa, D., Inada, T., Tanaka, M., Ueda, A., Kominami, G., Kambara, H., Kimura, T. and Kita, T. 2005. Serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels are elevated in acute coronary syndrome: a novel marker for early diagnosis. *Circulation.* **112**(6), pp.812-818.

Hevonoja, T., Pentikäinen, M.O., Hyvönen, M.T., Kovanen, P.T. and Ala-Korpela, M. 2000. Structure of low density lipoprotein (LDL) particles: Basis for understanding molecular changes in modified LDL. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids.* **1488**(3), pp.189-210.

Hiatt, W.R., Armstrong, E.J., Larson, C.J. and Brass, E.P. 2015. Pathogenesis of the Limb Manifestations and Exercise Limitations in Peripheral Artery Disease. *Circulation Research.* **116**(9), pp.1527-1539.

Hinagata, J., Kakutani, M., Fujii, T., Naruko, T., Inoue, N., Fujita, Y., Mehta, J.L., Ueda, M. and Sawamura, T. 2006. Oxidized LDL receptor LOX-1 is involved in neointimal hyperplasia after balloon arterial injury in a rat model. *Cardiovasc Res.* **69**(1), pp.263-271.

Homocysteine Studies, C. 2002. Homocysteine and risk of ischemic heart disease and stroke: a meta-analysis. *JAMA.* **288**(16), pp.2015-2022.

Honjo, M., Nakamura, K., Yamashiro, K., Kiryu, J., Tanihara, H., McEvoy, L.M., Honda, Y., Butcher, E.C., Masaki, T. and Sawamura, T. 2003. Lectin-like oxidized LDL receptor-1 is a cell-adhesion molecule involved in endotoxin-induced inflammation. *Proc Natl Acad Sci U S A.* **100**(3), pp.1274-1279.

Hoshikawa, H., Sawamura, T., Kakutani, M., Aoyama, T., Nakamura, T. and Masaki, T. 1998. High Affinity Binding of Oxidized LDL to Mouse Lectin-like Oxidized LDL Receptor (LOX-1). *Biochemical and Biophysical Research Communications.* **245**(3), pp.841-846.

Howard, B.V., Robbins, D.C., Sievers, M.L., Lee, E.T., Rhoades, D., Devereux, R.B., Cowan, L.D., Gray, R.S., Welty, T.K., Go, O.T. and Howard, W.J. 2000. LDL Cholesterol as a Strong Predictor of Coronary Heart Disease in Diabetic Individuals With Insulin Resistance and Low LDL. *The Strong Heart Study.* **20**(3), pp.830-835.

Hsieh, C.-C., Yen, M.-H., Yen, C.-H. and Lau, Y.-T. 2001. Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. *Cardiovascular Research.* **49**(1), pp.135-145.

Hu, C., Dandapat, A., Sun, L., Chen, J., Marwali, M.R., Romeo, F., Sawamura, T. and Mehta, J.L. 2008. LOX-1 deletion decreases collagen accumulation in atherosclerotic plaque in low-density lipoprotein receptor knockout mice fed a high-cholesterol diet. *Cardiovasc Res.* **79**(2), pp.287-293.

Hughes, D.J., Tiede, C., Penswick, N., Tang, A.A.S., Trinh, C.H., Mandal, U., Zajac, K.Z., Gaule, T., Howell, G., Edwards, T.A., Duan, J., Feyfant, E., McPherson, M.J., Tomlinson, D.C. and Whitehouse, A. 2017. Generation of specific inhibitors of SUMO1- and SUMO2/3-mediated protein-protein interactions using Affimer (Adhiron) technology. *Science signaling.* **10**(505), peaaj2005.

Ilkhani, H., Ravalli, A. and Marrazza, G. 2016. Design of an Affibody-Based Recognition Strategy for Human Epidermal Growth Factor Receptor 2 (HER2) Detection by Electrochemical Biosensors. *Chemosensors.* **4**(4), p23.

Inoue, N., Okamura, T., Kokubo, Y., Fujita, Y., Sato, Y., Nakanishi, M., Yanagida, K., Kakino, A., Iwamoto, S., Watanabe, M., Ogura, S., Otsui, K., Matsuda, H., Uchida, K., Yoshimoto, R. and

Sawamura, T. 2010. LOX index, a novel predictive biochemical marker for coronary heart disease and stroke. *Clin Chem.* **56**(4), pp.550-558.

Ishikawa, M., Ito, H., Akiyoshi, M., Kume, N., Yoshitomi, H., Mitsuoka, H., Tanida, S., Murata, K., Shibuya, H., Kasahara, T., Kakino, A., Fujita, Y., Sawamura, T., Yasuda, T. and Nakamura, T. 2012. Lectin-like oxidized low-density lipoprotein receptor 1 signal is a potent biomarker and therapeutic target for human rheumatoid arthritis. *Arthritis Rheum.* **64**(4), pp.1024-1034.

Jain, A., Subhan, I. and Joshi, M. 2009. Comparison of the point-of-care blood gas analyzer versus the laboratory auto-analyzer for the measurement of electrolytes. *International Journal of Emergency Medicine.* **2**(2), pp.117-120.

Jonasson, L., Holm, J., Skalli, O., Bondjers, G. and Hansson, G.K. 1986. Regional accumulations of T cells, macrophages, and smooth muscle cells in the human atherosclerotic plaque. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **6**(2), pp.131-138.

Jono, T., Miyazaki, A., Nagai, R., Sawamura, T., Kitamura, T. and Horiuchi, S. 2002. Lectin-like oxidized low density lipoprotein receptor-1 (LOX-1) serves as an endothelial receptor for advanced glycation end products (AGE). *FEBS Lett.* **511**(1-3), pp.170-174.

Kaboord, B. and Perr, M. 2008. Isolation of proteins and protein complexes by immunoprecipitation. *Methods Mol Biol.* **424**, pp.349-364.

Kakutani, M., Masaki, T. and Sawamura, T. 2000. A platelet–endothelium interaction mediated by lectin-like oxidized low-density lipoprotein receptor-1. *Proceedings of the National Academy of Sciences of the United States of America.* **97**(1), pp.360-364.

Kang, B.Y., Wang, W., Palade, P., Sharma, S.G. and Mehta, J.L. 2009. Cardiac hypertrophy during hypercholesterolemia and its amelioration with rosuvastatin and amlodipine. *J Cardiovasc Pharmacol.* **54**(4), pp.327-334.

Kannel, W.B., Dawber, T.R., Kagan, A., Revotskie, N. and Stokes, J., 3rd. 1961. Factors of risk in the development of coronary heart disease--six year follow-up experience. The Framingham Study. *Ann Intern Med.* **55**, pp.33-50.

Karon, B.S., Scott, R., Burritt, M.F. and Santrach, P.J. 2007. Comparison of lactate values between point-of-care and central laboratory analyzers. *Am J Clin Pathol.* **128**(1), pp.168-171.

Kataoka, H., Kume, N., Miyamoto, S., Minami, M., Morimoto, M., Hayashida, K., Hashimoto, N. and Kita, T. 2001. Oxidized LDL modulates Bax/Bcl-2 through the lectinlike Ox-LDL receptor-1 in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* **21**(6), pp.955-960.

Kataoka, H., Kume, N., Miyamoto, S., Minami, M., Moriwaki, H., Murase, T., Sawamura, T., Masaki, T., Hashimoto, N. and Kita, T. 1999. Expression of lectinlike oxidized low-density lipoprotein receptor-1 in human atherosclerotic lesions. *Circulation.* **99**(24), pp.3110-3117.

Khan, B.V., Parthasarathy, S.S., Alexander, R.W. and Medford, R.M. 1995. Modified low density lipoprotein and its constituents augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J Clin Invest.* **95**(3), pp.1262-1270.

King, R., Tiede, C., Simmons, K., Fishwick, C., Tomlinson, D. and Ajjan, R. 2015. Inhibition of complement C3 and fibrinogen interaction: a potential novel therapeutic target to reduce cardiovascular disease in diabetes. *Lancet.* **385 Suppl 1**, pS57.

Kobayashi, N., Hata, N., Kume, N., Seino, Y., Inami, T., Yokoyama, S., Shinada, T., Tomita, K., Kaneshige, T. and Mizuno, K. 2011. Soluble lectin-like oxidized low-density lipoprotein receptor-1 as an early biomarker for ST elevation myocardial infarction: time-dependent comparison with other biomarkers: time-dependent comparison with other biomarkers. *Circ J.* **75**(6), pp.1433-1439.

Kodama, T., Freeman, M., Rohrer, L., Zabrecky, J., Matsudaira, P. and Krieger, M. 1990. Type I macrophage scavenger receptor contains alpha-helical and collagen-like coiled coils. *Nature.* **343**(6258), pp.531-535.

Koide, A. and Koide, S. 2007. Monobodies: antibody mimics based on the scaffold of the fibronectin type III domain. *Methods Mol Biol.* **352**, pp.95-109.

Kolodych, S., Koniev, O., Baatarkhuu, Z., Bonnefoy, J.-Y., Debaene, F., Cianférani, S., Van Dorsselaer, A. and Wagner, A. 2015. CBTF: New Amine-to-Thiol Coupling Reagent for Preparation of Antibody Conjugates with Increased Plasma Stability. *Bioconjugate Chemistry.* **26**(2), pp.197-200.

Kuchibhotla, S., Vanegas, D., Kennedy, D.J., Guy, E., Nimako, G., Morton, R.E. and Febbraio, M. 2008. Absence of CD36 protects against atherosclerosis in ApoE knock-out mice with no

additional protection provided by absence of scavenger receptor A I/II. *Cardiovasc Res.* **78**(1), pp.185-196.

Kume, N., Mitsuoka, H., Hayashida, K., Tanaka, M. and Kita, T. 2010. Soluble lectin-like oxidized low-density lipoprotein receptor-1 predicts prognosis after acute coronary syndrome--a pilot study. *Circ J.* **74**(7), pp.1399-1404.

Kume, N., Mitsuoka, H., Hayashida, K., Tanaka, M., Kominami, G. and Kita, T. 2010. Soluble lectin-like oxidized LDL receptor-1 (sLOX-1) as a sensitive and specific biomarker for acute coronary syndrome—Comparison with other biomarkers. *Journal of Cardiology.* **56**(2), pp.159-165.

Kume, N., Murase, T., Moriwaki, H., Aoyama, T., Sawamura, T., Masaki, T. and Kita, T. 1998. Inducible expression of lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res.* **83**(3), pp.322-327.

Kyle, H.F., Wickson, K.F., Stott, J., Burslem, G.M., Breeze, A.L., Tiede, C., Tomlinson, D.C., Warriner, S.L., Nelson, A., Wilson, A.J. and Edwards, T.A. 2015. Exploration of the HIF-1alpha/p300 interface using peptide and Adhiron phage display technologies. *Mol Biosyst.* **11**(10), pp.2738-2749.

Lakhin, A.V., Tarantul, V.Z. and Gening, L.V. 2013. Aptamers: Problems, Solutions and Prospects. *Acta Naturae.* **5**(4), pp.34-43.

Landsberger, M., Zhou, J., Wilk, S., Thaumüller, C., Pavlovic, D., Otto, M., Whynot, S., Hung, O., Murphy, M.F., Cerny, V., Felix, S.B. and Lehmann, C. 2010. Inhibition of lectin-like oxidized low-density lipoprotein receptor-1 reduces leukocyte adhesion within the intestinal microcirculation in experimental endotoxemia in rats. *Critical Care.* **14**(6), pp.R223-R223.

Lee, S.-C., Park, K., Han, J., Lee, J.-j., Kim, H.J., Hong, S., Heu, W., Kim, Y.J., Ha, J.-S., Lee, S.-G., Cheong, H.-K., Jeon, Y.H., Kim, D. and Kim, H.-S. 2012. Design of a binding scaffold based on variable lymphocyte receptors of jawless vertebrates by module engineering. *Proceedings of the National Academy of Sciences.* **109**(9), pp.3299-3304.

Lequin, R.M. 2005. Enzyme Immunoassay (EIA)/Enzyme-Linked Immunosorbent Assay (ELISA). *Clinical Chemistry.* **51**(12), pp.2415-2418.

Levitan, I., Volkov, S. and Subbaiah, P.V. 2010. Oxidized LDL: Diversity, Patterns of Recognition, and Pathophysiology. *Antioxidants & Redox Signaling.* **13**(1), pp.39-75.

Lewis, L.M., Graffeo, C., Crosley, P., Klausner, H.A., Clark, C.L., Frank, A., Miner, J., Iarrobino, R. and Chyung, Y. 2015. Ecallantide for the acute treatment of angiotensin-converting enzyme inhibitor-induced angioedema: a multicenter, randomized, controlled trial. *Ann Emerg Med.* **65**(2), pp.204-213.

Li, D. and Mehta, J.L. 2000. Upregulation of endothelial receptor for oxidized LDL (LOX-1) by oxidized LDL and implications in apoptosis of human coronary artery endothelial cells: evidence from use of antisense LOX-1 mRNA and chemical inhibitors. *Arterioscler Thromb Vasc Biol.* **20**(4), pp.1116-1122.

Li, D., Williams, V., Liu, L., Chen, H., Sawamura, T., Romeo, F. and Mehta, J.L. 2003. Expression of lectin-like oxidized low-density lipoprotein receptors during ischemia-reperfusion and its role in determination of apoptosis and left ventricular dysfunction. *J Am Coll Cardiol.* **41**(6), pp.1048-1055.

Li, L., Sawamura, T. and Renier, G. 2003. Glucose enhances endothelial LOX-1 expression: role for LOX-1 in glucose-induced human monocyte adhesion to endothelium. *Diabetes.* **52**(7), pp.1843-1850.

Libby, P., Ridker, P.M. and Hansson, G.K. 2011. Progress and challenges in translating the biology of atherosclerosis. *Nature.* **473**(7347), pp.317-325.

Lopata, A., Hughes, R., Tiede, C., Heissler, S.M., Sellers, J.R., Knight, P.J., Tomlinson, D. and Peckham, M. 2018. Affimer proteins for F-actin: novel affinity reagents that label F-actin in live and fixed cells. *Scientific Reports.* **8**(1), p6572.

Luppa, P.B., Sokoll, L.J. and Chan, D.W. 2001. Immunosensors—principles and applications to clinical chemistry. *Clinica Chimica Acta.* **314**(1–2), pp.1-26.

Lusis, A.J. 2000. Atherosclerosis. *Nature.* **407**(6801), pp.233-241.

Magi, B. and Liberatori, S. 2005. Immunoblotting techniques. *Methods Mol Biol.* **295**, pp.227-254.

Malyar, N., Fürstenberg, T., Wellmann, J., Meyborg, M., Lüders, F., Gebauer, K., Bunzemeier, H., Roeder, N. and Reinecke, H. 2013. Recent trends in morbidity and in-hospital outcomes

of in-patients with peripheral arterial disease: a nationwide population-based analysis. *European Heart Journal.* **34**(34), pp.2706-2714.

Mango, R., Clementi, F., Borgiani, P., Forleo, G.B., Federici, M., Contino, G., Giardina, E., Garza, L., Fahdi, I.E., Lauro, R., Mehta, J.L., Novelli, G. and Romeo, F. 2003. Association of single nucleotide polymorphisms in the oxidised LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction. *J Med Genet.* **40**(12), pp.933-936.

Mango, R., Predazzi, I.M., Romeo, F. and Novelli, G. 2011. LOX-1/LOXIN: the yin/yang of atheroscleorosis. *Cardiovasc Drugs Ther.* **25**(5), pp.489-494.

Manning-Tobin, J.J., Moore, K.J., Seimon, T.A., Bell, S.A., Sharuk, M., Alvarez-Leite, J.I., de Winther, M.P., Tabas, I. and Freeman, M.W. 2009. Loss of SR-A and CD36 activity reduces atherosclerotic lesion complexity without abrogating foam cell formation in hyperlipidemic mice. *Arterioscler Thromb Vasc Biol.* **29**(1), pp.19-26.

Marques, M.E., Mansur, A.A.P. and Mansur, H.S. 2013. Chemical functionalization of surfaces for building three-dimensional engineered biosensors. *Applied Surface Science.* **275**, pp.347-360.

Martin-Fuentes, P., Civeira, F., Recalde, D., Garcia-Otin, A.L., Jarauta, E., Marzo, I. and Cenarro, A. 2007. Individual variation of scavenger receptor expression in human macrophages with oxidized low-density lipoprotein is associated with a differential inflammatory response. *J Immunol.* **179**(5), pp.3242-3248.

Marulanda-Londoño, E. and Chaturvedi, S. 2016. Stroke due to large vessel atherosclerosis: Five new things. *Neurology: Clinical Practice.* **6**(3), pp.252-258.

Marzilli, M., Merz, C.N.B., Boden, W.E., Bonow, R.O., Capozza, P.G., Chilian, W.M., DeMaria, A.N., Guarini, G., Huqi, A., Morrone, D., Patel, M.R. and Weintraub, W.S. 2012. Obstructive Coronary Atherosclerosis and Ischemic Heart Disease: An Elusive Link! *Journal of the American College of Cardiology.* **60**(11), pp.951-956.

McKeigue, P.M., Miller, G.J. and Marmot, M.G. 1989. Coronary heart disease in south Asians overseas: a review. *J Clin Epidemiol.* **42**(7), pp.597-609.

McLaughlin, K., Jardine, A.G. and Moss, J.G. 2000. Renal artery stenosis. *BMJ.* **320**(7242), pp.1124-1127.

Md Sayed, A.S., Zhao, Z., Guo, L., Li, F., Deng, X., Deng, H., Xia, K. and Yang, T. 2014. Serum Lectin-Like Oxidized-Low Density Lipoprotein Receptor-1 and Adiponectin Levels Are Associated With Coronary Artery Disease Accompanied With Metabolic Syndrome. *Iranian Red Crescent Medical Journal.* **16**(8), pe12106.

Mehta, J.L., Khaidakov, M., Hermonat, P.L., Mitra, S., Wang, X., Novelli, G. and Sawamura, T. 2011. LOX-1: A New Target for Therapy for Cardiovascular Diseases. *Cardiovascular Drugs and Therapy.* **25**(5), p495.

Mehta, J.L., Sanada, N., Hu, C.P., Chen, J., Dandapat, A., Sugawara, F., Satoh, H., Inoue, K., Kawase, Y., Jishage, K., Suzuki, H., Takeya, M., Schnackenberg, L., Beger, R., Hermonat, P.L., Thomas, M. and Sawamura, T. 2007. Deletion of LOX-1 reduces atherogenesis in LDLR knockout mice fed high cholesterol diet. *Circ Res.* **100**(11), pp.1634-1642.

Meyer, R.A., Lampe, P.D., Malewicz, B., Baumann, W.J. and Johnson, R.G. 1991. Enhanced gap junction formation with LDL and apolipoprotein B. *Exp Cell Res.* **196**(1), pp.72-81.

Miao, Y., Chen, J. and Hu, Y. 2005. Electrodeposited nonconducting polytyramine for the development of glucose biosensors. *Anal Biochem.* **339**(1), pp.41-45.

Minami, M., Kume, N., Kataoka, H., Morimoto, M., Hayashida, K., Sawamura, T., Masaki, T. and Kita, T. 2000. Transforming growth factor-beta(1) increases the expression of lectin-like oxidized low-density lipoprotein receptor-1. *Biochem Biophys Res Commun.* **272**(2), pp.357-361.

Minami, M., Kume, N., Shimaoka, T., Kataoka, H., Hayashida, K., Akiyama, Y., Nagata, I., Ando, K., Nobuyoshi, M., Hanyuu, M., Komeda, M., Yonehara, S. and Kita, T. 2001. Expression of SR-PSOX, a novel cell-surface scavenger receptor for phosphatidylserine and oxidized LDL in human atherosclerotic lesions. *Arterioscler Thromb Vasc Biol.* **21**(11), pp.1796-1800.

Miscoria, S.A., Barrera, G.D. and Rivas, G.A. 2006. Glucose biosensors based on the immobilization of glucose oxidase and polytyramine on rodhinized glassy carbon and screen printed electrodes. *Sensors and Actuators B: Chemical.* **115**(1), pp.205-211.

Mitsuoka, H., Kume, N., Hayashida, K., Inui-Hayashiada, A., Aramaki, Y., Toyohara, M., Jinnai, T., Nishi, E. and Kita, T. 2009. Interleukin 18 stimulates release of soluble lectin-like oxidized LDL receptor-1 (sLOX-1). *Atherosclerosis.* **202**(1), pp.176-182.

Moore, K.J. and Freeman, M.W. 2006. Scavenger Receptors in Atherosclerosis. *Beyond Lipid Uptake.* **26**(8), pp.1702-1711.

Moore, K.J., Kunjathoor, V.V., Koehn, S.L., Manning, J.J., Tseng, A.A., Silver, J.M., McKee, M. and Freeman, M.W. 2005. Loss of receptor-mediated lipid uptake via scavenger receptor A or CD36 pathways does not ameliorate atherosclerosis in hyperlipidemic mice. *J Clin Invest.* **115**(8), pp.2192-2201.

Moullan, N., Mouchiroud, L., Wang, X., Ryu, D., Williams, E.G., Mottis, A., Jovaisaite, V., Frochaux, M.V., Quiros, P.M., Deplancke, B., Houtkooper, R.H. and Auwerx, J. 2015. Tetracyclines disturb mitochondrial function across eukaryotic models: a call for caution in biomedical research. *Cell reports.* pp.S2211-1247(2215)00180-00181 00110.01016/j.celrep.02015.00102.00034.

Mouratou, B., Schaeffer, F., Guilvout, I., Tello-Manigne, D., Pugsley, A.P., Alzari, P.M. and Pecorari, F. 2007. Remodeling a DNA-binding protein as a specific in vivo inhibitor of bacterial secretin PulD. *Proc Natl Acad Sci U S A.* **104**(46), pp.17983-17988.

Mross, K., Richly, H., Fischer, R., Scharr, D., Buchert, M., Stern, A., Gille, H., Audoly, L.P. and Scheulen, M.E. 2013. First-in-human phase I study of PRS-050 (Angiocal), an Anticalin targeting and antagonizing VEGF-A, in patients with advanced solid tumors. *PLoS One.* **8**(12), pe83232.

Murase, T., Kume, N., Kataoka, H., Minami, M., Sawamura, T., Masaki, T. and Kita, T. 2000. Identification of soluble forms of lectin-like oxidized LDL receptor-1. *Arterioscler Thromb Vasc Biol.* **20**(3), pp.715-720.

Murphy, J.E., Tedbury, P.R., Homer-Vanniasinkam, S., Walker, J.H. and Ponnambalam, S. 2005. Biochemistry and cell biology of mammalian scavenger receptors. *Atherosclerosis.* **182**(1), pp.1-15.

Murphy, J.E., Vohra, R.S., Dunn, S., Holloway, Z.G., Monaco, A.P., Homer-Vanniasinkam, S., Walker, J.H. and Ponnambalam, S. 2008. Oxidised LDL internalisation by the LOX-1 scavenger receptor is dependent on a novel cytoplasmic motif and is regulated by dynamin-2. *J Cell Sci.* **121**(Pt 13), pp.2136-2147.

Nagase, M., Kaname, S., Nagase, T., Wang, G., Ando, K., Sawamura, T. and Fujita, T. 2000. Expression of LOX-1, an oxidized low-density lipoprotein receptor, in experimental hypertensive glomerulosclerosis. *J Am Soc Nephrol.* **11**(10), pp.1826-1836.

Nakagawa, T., Akagi, M., Hoshikawa, H., Chen, M., Yasuda, T., Mukai, S., Ohsawa, K., Masaki, T., Nakamura, T. and Sawamura, T. 2002. Lectin-like oxidized low-density lipoprotein receptor 1 mediates leukocyte infiltration and articular cartilage destruction in rat zymosan-induced arthritis. *Arthritis & Rheumatism.* **46**(9), pp.2486-2494.

Nakano, A., Inoue, N., Sato, Y., Nishimichi, N., Takikawa, K., Fujita, Y., Kakino, A., Otsui, K., Yamaguchi, S., Matsuda, H. and Sawamura, T. 2010. LOX-1 mediates vascular lipid retention under hypertensive state. *J Hypertens.* **28**(6), pp.1273-1280.

National Institute for Health and Care Excellence. 2016. *Alirocumab for treating primary hypercholesterolaemia and mixed dyslipidaemia [TA393].* London.

Nixon, A.E. and Wood, C.R. 2006. Engineered protein inhibitors of proteases. *Curr Opin Drug Discov Devel.* **9**(2), pp.261-268.

Nygren, P.A. 2008. Alternative binding proteins: affibody binding proteins developed from a small three-helix bundle scaffold. *Febs j.* **275**(11), pp.2668-2676.

Ockene, I.S. and Miller, N.H. 1997. Cigarette Smoking, Cardiovascular Disease, and Stroke. *A Statement for Healthcare Professionals From the American Heart Association.* **96**(9), pp.3243-3247.

Ohki, I., Amida, H., Yamada, R., Sugihara, M., Ishigaki, T. and Tate, S. 2011. Surface plasmon resonance study on functional significance of clustered organization of lectin-like oxidized LDL receptor (LOX-1). *Biochim Biophys Acta.* **1814**(2), pp.345-354.

Ohki, I., Ishigaki, T., Oyama, T., Matsunaga, S., Xie, Q., Ohnishi-Kameyama, M., Murata, T., Tsuchiya, D., Machida, S., Morikawa, K. and Tate, S. 2005. Crystal structure of human lectin-like, oxidized low-density lipoprotein receptor 1 ligand binding domain and its ligand recognition mode to OxLDL. *Structure.* **13**(6), pp.905-917.

Ohtani, K., Suzuki, Y., Eda, S., Kawai, T., Kase, T., Keshi, H., Sakai, Y., Fukuoh, A., Sakamoto, T., Itabe, H., Suzutani, T., Ogasawara, M., Yoshida, I. and Wakamiya, N. 2001. The membrane-

type collectin CL-P1 is a scavenger receptor on vascular endothelial cells. *J Biol Chem.* **276**(47), pp.44222-44228.

Oka, K., Sawamura, T., Kikuta, K., Itokawa, S., Kume, N., Kita, T. and Masaki, T. 1998. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. *Proc Natl Acad Sci U S A.* **95**(16), pp.9535-9540.

Omland, T., Richards, A.M., Wergeland, R. and Vik-Mo, H. 2005. B-type natriuretic peptide and long-term survival in patients with stable coronary artery disease. *Am J Cardiol.* **95**(1), pp.24-28.

Ouellet, E., Yang, C.W.T., Lin, T., Yang, L.L. and Lagally, E.T. 2010. Novel Carboxyl-Amine Bonding Methods for Poly(dimethylsiloxane)-Based Devices. *Langmuir.* **26**(14), pp.11609-11614.

Ouerghi, O., Touhami, A., Jaffrezic-Renault, N., Martelet, C., Ouada, H.B. and Cosnier, S. 2002. Impedimetric immunosensor using avidin–biotin for antibody immobilization. *Bioelectrochemistry.* **56**(1), pp.131-133.

Palmieri, V.O., Coppola, B., Grattagliano, I., Casieri, V., Cardinale, G., Portincasa, P., Palasciano, G. and Di Serio, F. 2013. Oxidized LDL receptor 1 gene polymorphism in patients with metabolic syndrome. *Eur J Clin Invest.* **43**(1), pp.41-48.

Panel, Mohr, J.P., Albers, G.W., Amarenco, P., Babikian, V.L., Biller, J., Brey, R.L., Coull, B., Easton, J.D., Gomez, C.R., Helgason, C.M., Kase, C.S., Pullicino, P.M. and Turpie, A.G.G. 1997. Etiology of Stroke. *Stroke.* **28**(7), pp.1501-1506.

Parlato, S., Romagnoli, G., Spadaro, F., Canini, I., Sirabella, P., Borghi, P., Ramoni, C., Filesi, I., Biocca, S., Gabriele, L. and Belardelli, F. 2010. LOX-1 as a natural IFN-alpha-mediated signal for apoptotic cell uptake and antigen presentation in dendritic cells. *Blood.* **115**(8), pp.1554-1563.

Parthasarathy, S., Wieland, E. and Steinberg, D. 1989. A role for endothelial cell lipoxygenase in the oxidative modification of low density lipoprotein. *Proc Natl Acad Sci U S A.* **86**(3), pp.1046-1050.

Pirillo, A. and Catapano, A.L. 2013. Soluble lectin-like oxidized low density lipoprotein receptor-1 as a biochemical marker for atherosclerosis-related diseases. *Dis Markers.* **35**(5), pp.413-418.

PrabhuDas, M., Bowdish, D., Drickamer, K., Febbraio, M., Herz, J., Kobzik, L., Krieger, M., Loike, J., Means, T.K., Moestrup, S.K., Post, S., Sawamura, T., Silverstein, S., Wang, X.-Y. and El Khoury, J. 2014. Standardizing Scavenger Receptor Nomenclature. *Journal of immunology (Baltimore, Md. : 1950).* **192**(5), pp.1997-2006.

Public Health England. 2017. *Health Profile for England.* London.

Rajavashisth, T.B., Andalibi, A., Territo, M.C., Berliner, J.A., Navab, M., Fogelman, A.M. and Lusis, A.J. 1990. Induction of endothelial cell expression of granulocyte and macrophage colony-stimulating factors by modified low-density lipoproteins. *Nature.* **344**(6263), pp.254-257.

Ren, J., Grundy, S.M., Liu, J., Wang, W., Wang, M., Sun, J., Liu, J., Li, Y., Wu, Z. and Zhao, D. 2010. Long-term coronary heart disease risk associated with very-low-density lipoprotein cholesterol in Chinese: the results of a 15-Year Chinese Multi-Provincial Cohort Study (CMCS). *Atherosclerosis.* **211**(1), pp.327-332.

Renedo, O.D., Alonso-Lomillo, M.A. and Martínez, M.J.A. 2007. Recent developments in the field of screen-printed electrodes and their related applications. *Talanta.* **73**(2), pp.202-219.

Ridker, P.M. 2001. High-Sensitivity C-Reactive Protein. *Potential Adjunct for Global Risk Assessment in the Primary Prevention of Cardiovascular Disease.* **103**(13), pp.1813-1818.

Robinson, J.I., Baxter, E.W., Owen, R.L., Thomsen, M., Tomlinson, D.C., Waterhouse, M.P., Win, S.J., Nettleship, J.E., Tiede, C., Foster, R.J., Owens, R.J., Fishwick, C.W.G., Harris, S.A., Goldman, A., McPherson, M.J. and Morgan, A.W. 2017. Affimer proteins inhibit immune complex binding to FcγRIIIa with high specificity through competitive and allosteric modes of action. *Proceedings of the National Academy of Sciences.*

Roger, V.L., Go, A.S., Lloyd-Jones, D.M., Adams, R.J., Berry, J.D., Brown, T.M., Carnethon, M.R., Dai, S., de Simone, G., Ford, E.S., Fox, C.S., Fullerton, H.J., Gillespie, C., Greenlund, K.J., Hailpern, S.M., Heit, J.A., Ho, P.M., Howard, V.J., Kissela, B.M., Kittner, S.J., Lackland, D.T., Lichtman, J.H., Lisabeth, L.D., Makuc, D.M., Marcus, G.M., Marelli, A., Matchar, D.B., McDermott, M.M., Meigs, J.B., Moy, C.S., Mozaffarian, D., Mussolino, M.E., Nichol, G.,

Paynter, N.P., Rosamond, W.D., Sorlie, P.D., Stafford, R.S., Turan, T.N., Turner, M.B., Wong, N.D., Wylie-Rosett, J., on behalf of the American Heart Association Statistics, C. and Stroke Statistics, S. 2011. Heart Disease and Stroke Statistics—2011 Update: A Report From the American Heart Association. *Circulation.* **123**(4), pp.e18-e209.

Rogers, K.R. 2000. Principles of affinity-based biosensors. *Molecular Biotechnology.* **14**(2), pp.109-129.

Rogers, K.R. 2006. Recent advances in biosensor techniques for environmental monitoring. *Analytica Chimica Acta.* **568**(1), pp.222-231.

Ross, R. and Glomset, J.A. 1973. Atherosclerosis and the arterial smooth muscle cell: Proliferation of smooth muscle is a key event in the genesis of the lesions of atherosclerosis. *Science.* **180**(4093), pp.1332-1339.

Ryoo, S., Bhunia, A., Chang, F., Shoukas, A., Berkowitz, D.E. and Romer, L.H. 2011. OxLDL-dependent activation of arginase II is dependent on the LOX-1 receptor and downstream RhoA signaling. *Atherosclerosis.* **214**(2), pp.279-287.

Sabatine, M.S., Giugliano, R.P., Keech, A.C., Honarpour, N., Wiviott, S.D., Murphy, S.A., Kuder, J.F., Wang, H., Liu, T., Wasserman, S.M., Sever, P.S. and Pedersen, T.R. 2017. Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease. *New England Journal of Medicine.* **376**(18), pp.1713-1722.

Savenkova, M.L., Mueller, D.M. and Heinecke, J.W. 1994. Tyrosyl radical generated by myeloperoxidase is a physiological catalyst for the initiation of lipid peroxidation in low density lipoprotein. *J Biol Chem.* **269**(32), pp.20394-20400.

Sawamura, T., Kume, N., Aoyama, T., Moriwaki, H., Hoshikawa, H., Aiba, Y., Tanaka, T., Miwa, S., Katsura, Y., Kita, T. and Masaki, T. 1997. An endothelial receptor for oxidized low-density lipoprotein. *Nature.* **386**(6620), pp.73-77.

Schiff, D., Kesari, S., de Groot, J., Mikkelsen, T., Drappatz, J., Coyle, T., Fichtel, L., Silver, B., Walters, I. and Reardon, D. 2015. Phase 2 study of CT-322, a targeted biologic inhibitor of VEGFR-2 based on a domain of human fibronectin, in recurrent glioblastoma. *Invest New Drugs.* **33**(1), pp.247-253.

Schlichthaerle, T., Eklund, A.S., Schueder, F., Strauss, M.T., Tiede, C., Curd, A., Ries, J., Peckham, M., Tomlinson, D.C. and Jungmann, R. 2018. Site-Specific Labeling of Affimers for DNA-PAINT Microscopy. *Angewandte Chemie International Edition.* **0**(0).

Scott, A.M., Allison, J.P. and Wolchok, J.D. 2012. Monoclonal antibodies in cancer therapy. *Cancer Immunity.* **12**, p14.

Segrest, J.P., Jones, M.K., De Loof, H. and Dashti, N. 2001. Structure of apolipoprotein B-100 in low density lipoproteins. *Journal of Lipid Research.* **42**(9), pp.1346-1367.

Sentinelli, F., Filippi, E., Fallarino, M., Romeo, S., Fanelli, M., Buzzetti, R., Berni, A. and Baroni, M.G. 2006. The 3'-UTR C>T polymorphism of the oxidized LDL-receptor 1 (OLR1) gene does not associate with coronary artery disease in Italian CAD patients or with the severity of coronary disease. *Nutr Metab Cardiovasc Dis.* **16**(5), pp.345-352.

Shah, B. and Mayer, L. 2010. Current status of monoclonal antibody therapy for the treatment of inflammatory bowel disease. *Expert review of clinical immunology.* **6**(4), pp.607-620.

Shannahan, J.H., Bai, W. and Brown, J.M. 2015. Implications of scavenger receptors in the safe development of nanotherapeutics. *Receptors & clinical investigation.* **2**(3), pe811.

Shimaoka, T., Kume, N., Minami, M., Hayashida, K., Kataoka, H., Kita, T. and Yonehara, S. 2000. Molecular Cloning of a Novel Scavenger Receptor for Oxidized Low Density Lipoprotein, SR-PSOX, on Macrophages. *Journal of Biological Chemistry.* **275**(52), pp.40663-40666.

Shimaoka, T., Kume, N., Minami, M., Hayashida, K., Sawamura, T., Kita, T. and Yonehara, S. 2001. LOX-1 supports adhesion of Gram-positive and Gram-negative bacteria. *J Immunol.* **166**(8), pp.5108-5114.

Silverman, J., Lu, Q., Bakker, A., To, W., Duguay, A., Alba, B.M., Smith, R., Rivas, A., Li, P., Le, H., Whitehorn, E., Moore, K.W., Swimmer, C., Perlroth, V., Vogt, M., Kolkman, J. and Stemmer, W.P.C. 2005. Multivalent avimer proteins evolved by exon shuffling of a family of human receptor domains. *Nat Biotech.* **23**(12), pp.1556-1561.

Situmorang, M., Gooding, J.J., Hibbert, D.B. and Barnett, D. 1998. Electrodeposited polytyramine as an immobilisation matrix for enzyme biosensors. *Biosens Bioelectron.* **13**(9), pp.953-962.

Skerra, A. 2008. Alternative binding proteins: anticalins - harnessing the structural plasticity of the lipocalin ligand pocket to engineer novel binding activities. *Febs j.* **275**(11), pp.2677-2683.

Smithwick, E. and Stewart, M.W. 2017. Designed Ankyrin Repeat Proteins: A look at their evolving use in medicine with a focus on the treatment of chorioretinal vascular disorders. *Antiinflamm Antiallergy Agents Med Chem.*

Snowden, C.B., McNamara, P.M., Garrison, R.J., Feinleib, M., Kannel, W.B. and Epstein, F.H. 1982. Predicting coronary heart disease in siblings--a multivariate assessment: the Framingham Heart Study. *Am J Epidemiol.* **115**(2), pp.217-222.

Song, S., Wang, L., Li, J., Fan, C. and Zhao, J. 2008. Aptamer-based biosensors. *TrAC Trends in Analytical Chemistry.* **27**(2), pp.108-117.

Sreenarasimhaiah, J. 2005. Chronic mesenteric ischemia. *Best Pract Res Clin Gastroenterol.* **19**(2), pp.283-295.

Stack, E.C., Wang, C., Roman, K.A. and Hoyt, C.C. 2014. Multiplexed immunohistochemistry, imaging, and quantitation: A review, with an assessment of Tyramide signal amplification, multispectral imaging and multiplex analysis. *Methods.* **70**(1), pp.46-58.

Stary, H.C. 2000. Natural History and Histological Classification of Atherosclerotic Lesions. *An Update.* **20**(5), pp.1177-1178.

Stary, H.C., Blankenhorn, D.H., Chandler, A.B., Glagov, S., Insull, W., Richardson, M., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J. and Wagner, W.D. 1992. A definition of the intima of human arteries and of its atherosclerosis-prone regions. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation.* **85**(1), pp.391-405.

Stary, H.C., Chandler, A.B., Glagov, S., Guyton, J.R., Insull, W., Rosenfeld, M.E., Schaffer, S.A., Schwartz, C.J., Wagner, W.D. and Wissler, R.W. 1994. A definition of initial, fatty streak, and intermediate lesions of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **14**(5), pp.840-856.

Steemson, J.D., Baake, M., Rakonjac, J., Arcus, V.L. and Liddament, M.T. 2014. Tracking molecular recognition at the atomic level with a new protein scaffold based on the OB-fold. *PLoS One.* **9**(1), pe86050.

Steinbrecher, U.P. 1988. Role of superoxide in endothelial-cell modification of low-density lipoproteins. *Biochim Biophys Acta.* **959**(1), pp.20-30.

Straw, S., Ferrigno, P., Song, Q., Tomlinson, D. and Galdo, F. 2013. Proof of concept study to identify candidate biomarkers of fibrosis using high throughput peptide aptamer microarray and validate by enzyme linked immunosorbant assay. *Journal of Biomedical Science and Engineering.* **6**(8B), pp.32-42.

Stumpp, M.T., Binz, H.K. and Amstutz, P. 2008. DARPins: a new generation of protein therapeutics. *Drug Discov Today.* **13**(15-16), pp.695-701.

Sun, L., Ishida, T., Yasuda, T., Kojima, Y., Honjo, T., Yamamoto, Y., Yamamoto, H., Ishibashi, S., Hirata, K.-i. and Hayashi, Y. 2009. RAGE mediates oxidized LDL-induced pro-inflammatory effects and atherosclerosis in non-diabetic LDL receptor-deficient mice. *Cardiovascular Research.* **82**(2), pp.371-381.

Tabas, I., Williams, K.J. and Borén, J. 2007. Subendothelial Lipoprotein Retention as the Initiating Process in Atherosclerosis. *Update and Therapeutic Implications.* **116**(16), pp.1832-1844.

Taleat, Z., Khoshroo, A. and Mazloum-Ardakani, M. 2014. Screen-printed electrodes for biosensing: a review (2008–2013). *Microchimica Acta.* **181**(9), pp.865-891.

Tamura, Y., Adachi, H., Osuga, J., Ohashi, K., Yahagi, N., Sekiya, M., Okazaki, H., Tomita, S., Iizuka, Y., Shimano, H., Nagai, R., Kimura, S., Tsujimoto, M. and Ishibashi, S. 2003. FEEL-1 and FEEL-2 are endocytic receptors for advanced glycation end products. *J Biol Chem.* **278**(15), pp.12613-12617.

Tamura, Y., Osuga, J., Adachi, H., Tozawa, R., Takanezawa, Y., Ohashi, K., Yahagi, N., Sekiya, M., Okazaki, H., Tomita, S., Iizuka, Y., Koizumi, H., Inaba, T., Yagyu, H., Kamada, N., Suzuki, H., Shimano, H., Kadowaki, T., Tsujimoto, M., Arai, H., Yamada, N. and Ishibashi, S. 2004. Scavenger receptor expressed by endothelial cells I (SREC-I) mediates the uptake of acetylated low density lipoproteins by macrophages stimulated with lipopolysaccharide. *J Biol Chem.* **279**(30), pp.30938-30944.

Tan, K.C., Shiu, S.W., Wong, Y., Leng, L. and Bucala, R. 2008. Soluble lectin-like oxidized low density lipoprotein receptor-1 in type 2 diabetes mellitus. *J Lipid Res.* **49**(7), pp.1438-1444.

Tanaka, T., Hishitani, Y. and Ogata, A. 2014. Monoclonal antibodies in rheumatoid arthritis: comparative effectiveness of tocilizumab with tumor necrosis factor inhibitors. *Biologics : Targets & Therapy.* **8**, pp.141-153.

Tatsuguchi, M., Furutani, M., Hinagata, J., Tanaka, T., Furutani, Y., Imamura, S., Kawana, M., Masaki, T., Kasanuki, H., Sawamura, T. and Matsuoka, R. 2003. Oxidized LDL receptor gene (OLR1) is associated with the risk of myocardial infarction. *Biochem Biophys Res Commun.* **303**(1), pp.247-250.

Thakkar, S., Wang, X., Khaidakov, M., Dai, Y., Gokulan, K., Mehta, J.L. and Varughese, K.I. 2015. Structure-based Design Targeted at LOX-1, a Receptor for Oxidized Low-Density Lipoprotein. *Scientific Reports.* **5**, p16740.

Tiede, Bedford, R., Heseltine, S.J., Smith, G., Wijetunga, I., Ross, R., AlQallaf, D., Roberts, A.P., Balls, A., Curd, A., Hughes, R.E., Martin, H., Needham, S.R., Zanetti-Domingues, L.C., Sadigh, Y., Peacock, T.P., Tang, A.A., Gibson, N., Kyle, H., Platt, G.W., Ingram, N., Taylor, T., Coletta, L.P., Manfield, I., Knowles, M., Bell, S., Esteves, F., Maqbool, A., Prasad, R.K., Drinkhill, M., Bon, R.S., Patel, V., Goodchild, S.A., Martin-Fernandez, M., Owens, R.J., Nettleship, J.E., Webb, M.E., Harrison, M., Lippiat, J.D., Ponnambalam, S., Peckham, M., Smith, A., Ferrigno, P.K., Johnson, M., McPherson, M.J. and Tomlinson, D.C. 2017. Affimer proteins are versatile and renewable affinity reagents. *Elife.* **6**.

Tiede, C., Bedford, R., Heseltine, S.J., Smith, G., Wijetunga, I., Ross, R., AlQallaf, D., Roberts, A.P.E., Balls, A., Curd, A., Hughes, R.E., Martin, H., Needham, S.R., Zanetti-Domingues, L.C., Sadigh, Y., Peacock, T.P., Tang, A.A., Gibson, N., Kyle, H., Platt, G.W., Ingram, N., Taylor, T., Coletta, L.P., Manfield, I., Knowles, M., Bell, S., Esteves, F., Maqbool, A., Prasad, R.K., Drinkhill, M., Bon, R.S., Patel, V., Goodchild, S.A., Martin-Fernandez, M., Owens, R.J., Nettleship, J.E., Webb, M.E., Harrison, M., Lippiat, J.D., Ponnambalam, S., Peckham, M., Smith, A., Ferrigno, P.K., Johnson, M., McPherson, M.J. and Tomlinson, D.C. 2017. Affimer proteins are versatile and renewable affinity reagents. *eLife.* **6**, pe24903.

Tiede, C., Tang, A.A., Deacon, S.E., Mandal, U., Nettleship, J.E., Owen, R.L., George, S.E., Harrison, D.J., Owens, R.J., Tomlinson, D.C. and McPherson, M.J. 2014. Adhiron: a stable and

versatile peptide display scaffold for molecular recognition applications. *Protein Eng Des Sel.* **27**(5), pp.145-155.

Ueno, T., Kaname, S., Takaichi, K., Nagase, M., Tojo, A., Onozato, M.L. and Fujita, T. 2003. LOX-1, an oxidized low-density lipoprotein receptor, was upregulated in the kidneys of chronic renal failure rats. *Hypertens Res.* **26**(1), pp.117-122.

van der Wal, A.C. and Becker, A.E. 1999. Atherosclerotic plaque rupture--pathologic basis of plaque stability and instability. *Cardiovasc Res.* **41**(2), pp.334-344.

Varadamsetty, G., Tremmel, D., Hansen, S., Parmeggiani, F. and Pluckthun, A. 2012. Designed Armadillo repeat proteins: library generation, characterization and selection of peptide binders with high specificity. *J Mol Biol.* **424**(1-2), pp.68-87.

Vasan, R.S. 2006. Biomarkers of Cardiovascular Disease. *Molecular Basis and Practical Considerations.* **113**(19), pp.2335-2362.

Vericat, C., Vela, M.E., Benitez, G., Carro, P. and Salvarezza, R.C. 2010. Self-assembled monolayers of thiols and dithiols on gold: new challenges for a well-known system. *Chemical Society Reviews.* **39**(5), pp.1805-1834.

Veronese, F.M. and Mero, A. 2008. The impact of PEGylation on biological therapies. *BioDrugs.* **22**(5), pp.315-329.

Vincent, A.M., Hayes, J.M., McLean, L.L., Vivekanandan-Giri, A., Pennathur, S. and Feldman, E.L. 2009. Dyslipidemia-Induced Neuropathy in Mice: The Role of oxLDL/LOX-1. *Diabetes.* **58**(10), pp.2376-2385.

Vinores, S.A. 2006. Pegaptanib in the treatment of wet, age-related macular degeneration. *International Journal of Nanomedicine.* **1**(3), pp.263-268.

Vlodaver, Z., Neufeld, H.N. and Edwards, J.E. 1972. Pathology of Angina Pectoris. *Circulation.* **46**(6), pp.1048-1064.

Vohra, R.S., Murphy, J.E., Walker, J.H., Homer-Vanniasinkam, S. and Ponnambalam, S. 2007. Functional refolding of a recombinant C-type lectin-like domain containing intramolecular disulfide bonds. *Protein Expr Purif.* **52**(2), pp.415-421.

Wågsäter, D., Olofsson, P.S., Norgren, L., Stenberg, B. and Sirsjö, A. 2004. The chemokine and scavenger receptor CXCL16/SR-PSOX is expressed in human vascular smooth muscle cells and is induced by interferon γ. *Biochemical and Biophysical Research Communications.* **325**(4), pp.1187-1193.

Wang, X., Phillips, M.I. and Mehta, J.L. 2011. LOX-1 and angiotensin receptors, and their interplay. *Cardiovasc Drugs Ther.* **25**(5), pp.401-417.

White, S.J., Sala-Newby, G.B. and Newby, A.C. 2011. Overexpression of scavenger receptor LOX-1 in endothelial cells promotes atherogenesis in the ApoE(-/-) mouse model. *Cardiovasc Pathol.* **20**(6), pp.369-373.

Wilcken, D.E. and Wilcken, B. 1976. The pathogenesis of coronary artery disease. A possible role for methionine metabolism. *J Clin Invest.* **57**(4), pp.1079-1082.

Wild, S. and McKeigue, P. 1997. Cross sectional analysis of mortality by country of birth in England and Wales, 1970-92. *Bmj.* **314**(7082), pp.705-710.

Wilkins E, W.L., Wickramasinghe K, Bhatnagar P, Leal J, Luengo-Fernandez R, Burns R, Rayner M, Townsend N 2017. *European Cardiovascular Disease Statistics 2017.* Brussels: European Heart Network.

Williams, K.J. and Tabas, I. 2005. Lipoprotein Retention—and Clues for Atheroma Regression. *Arteriosclerosis, Thrombosis, and Vascular Biology.* **25**(8), pp.1536-1540.

Witztum, J.L. 1994. The oxidation hypothesis of atherosclerosis. *Lancet.* **344**(8925), pp.793-795.

Woywodt, A., Bahlmann, F.H., De Groot, K., Haller, H. and Haubitz, M. 2002. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. *Nephrol Dial Transplant.* **17**(10), pp.1728-1730.

Wu, Z., Sawamura, T., Kurdowska, A.K., Ji, H.-L., Idell, S. and Fu, J. 2011. LOX-1 Deletion Improves Neutrophil Responses, Enhances Bacterial Clearance, and Reduces Lung Injury in a Murine Polymicrobial Sepsis Model. *Infection and Immunity.* **79**(7), pp.2865-2870.

Xie, C., Tiede, C., Zhang, X., Wang, C., Li, Z., Xu, X., McPherson, M.J., Tomlinson, D.C. and Xu, W. 2017. Development of an Affimer-antibody combined immunological diagnosis kit for glypican-3. *Scientific Reports.* **7**(1), p9608.

Xu, C.X., Marzouk, S.A.M., Cosofret, V.V., Buck, R.P., Neuman, M.R. and Sprinkle, R.H. 1997. Development of a diamine biosensor. *Talanta.* **44**(9), pp.1625-1632.

Xu, S., Jay, A., Brunaldi, K., Huang, N. and Hamilton, J.A. 2013. CD36 enhances fatty acid uptake by increasing the rate of intracellular esterification but not transport across the plasma membrane. *Biochemistry.* **52**(41), pp.7254-7261.

Xu, X., Gao, X., Potter, B.J., Cao, J.M. and Zhang, C. 2007. Anti-LOX-1 rescues endothelial function in coronary arterioles in atherosclerotic ApoE knockout mice. *Arterioscler Thromb Vasc Biol.* **27**(4), pp.871-877.

Yalow, R.S. and Berson, S.A. 1960. Immunoassay of endogenous plasma insulin in man. *J Clin Invest.* **39**, pp.1157-1175.

Yamanaka, K., Vestergaard, M.C. and Tamiya, E. 2016. Printable Electrochemical Biosensors: A Focus on Screen-Printed Electrodes and Their Application. *Sensors (Basel).* **16**(10).

Yamanaka, S., Zhang, X.Y., Miura, K., Kim, S. and Iwao, H. 1998. The human gene encoding the lectin-type oxidized LDL receptor (OLR1) is a novel member of the natural killer gene complex with a unique expression profile. *Genomics.* **54**(2), pp.191-199.

Yoshida, H., Kondratenko, N., Green, S., Steinberg, D. and Quehenberger, O. 1998. Identification of the lectin-like receptor for oxidized low-density lipoprotein in human macrophages and its potential role as a scavenger receptor. *Biochem J.* **334 (Pt 1)**, pp.9-13.

Young, I.S. and McEneny, J. 2001. Lipoprotein oxidation and atherosclerosis. *Biochemical Society Transactions.* **29**(2), pp.358-362.

Zhang, H., Ma, G., Yao, Y., Qian, H., Li, W., Chen, X., Jiang, W. and Zheng, R. 2012. Olmesartan Attenuates the Impairment of Endothelial Cells Induced by Oxidized Low Density Lipoprotein through Downregulating Expression of LOX-1. *International Journal of Molecular Sciences.* **13**(2), pp.1512-1523.

Zhang, J., Yin, C., Zhang, Y., Zhao, L., Fu, H. and Feng, J. 2013. The role of OLR1 polymorphisms in determining the risk and prognosis of ischemic stroke in a Chinese population. *NeuroRehabilitation.* **32**(2), pp.391-396.

Zhurauski, P., Arya, S.K., Jolly, P., Tiede, C., Tomlinson, D.C., Ko Ferrigno, P. and Estrela, P. 2018. Sensitive and selective Affimer-functionalised interdigitated electrode-based

capacitive biosensor for Her4 protein tumour biomarker detection. *Biosensors and Bioelectronics.* **108**, pp.1-8.