Thesis for the award of Master of Science

Novel polymers to encourage rapid recovery of arterial function following coronary stenting

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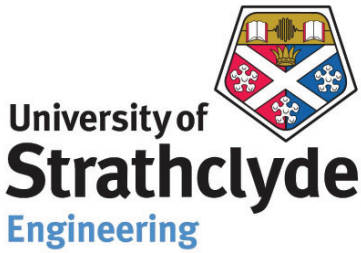
Tuesday 14th August 2012



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This thesis is the result of the author's original research. It has been composed by the author and has not been previously submitted for examination which has led to the award of a degree.

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Contents

Abstract

Intravascular stenting in conjunction with balloon angioplasty is standard practice in percutaneous coronary interventions. Despite widespread acceptance of this procedure associated limitations remain, notably in-stent restenosis, delayed vessel healing and late-stent thrombosis. Normal arterial function is preserved in a healthy coronary artery by the endothelium, a single layer of cells which lines the lumen protecting against thrombus formation and helping to control vascular tone. Following coronary stenting the endothelium is destroyed, exposing the underlying smooth muscle cells to a powerful inflammatory stimulus. This can cause smooth muscle cell hyperplasia which thickens the neointima thus narrowing the lumen of the artery, which may then require repeat revascularisation.

Existing Drug-Eluting Stents (DES) release cytostatic drugs which inhibit smooth muscle cell proliferation. Despite positive indications from initial clinical trials, an associated morbidity has emerged attributed to impaired re-endothelialisation due to negative effects of eluted drugs and the unfavourable growth environment provided by the polymer.

This project investigates polypyrrole, a non-toxic conducting polymer, as a novel coating more conducive to re-endothelialisation for use in a next generation DES. Porcine primary endothelial and smooth muscle cells, harvested and cultured in-vitro on a series of polypyrrole surfaces, were assessed in terms of confluence, viability, adherence, morphology and migration. Polypyrrole was synthesised by electropolymerisation onto medical grade stainless steel plates.

In these circumstances topography remained consistent despite variation in synthesis techniques. However, an apparent influence remained of the polypyrrole and dopant drug on cell growth and morphology. Proliferation of endothelial and smooth muscle cells, displaying characteristic morphologies, was observed on polypyrrole, with low LDH levels indicating a high level of viability. This preliminary study demonstrates the considerable potential displayed by polypyrrole for use in a vascular environment and supports further research into the development of a polypyrrole-based drug-polymer for use in coronary stenting.

1. Background

1.1. The coronary artery in health and disease

A healthy artery is lined by a single layer of endothelial cells (EC), forming the endothelium, or tunica intima. These cells are fundamental in maintaining a healthy artery. They prevent blood from clotting in the circulation, even when blood flow is minimal, and help to maintain vascular tone. The endothelium rests on the internal elastic lamina, underneath which are the smooth muscle cells (SMC) which are the main component of the medial layer of the vessel wall. An external elastic lamina lies between the medial layer and the adventia, which is the outermost layer of the artery. The inner adventitia is made up of sheets of densely interwoven collagen fibres, elastin fibres and fibroblasts, moving to a tangled and collagen-rich arrangement towards the outer reaches. The disordered arrangement of the fibres means that the tissue can respond well to axial loading, becoming stronger as load increases and fibres are pulled taut [10].

In coronary heart disease (CHD), the lumen of the coronary arteries narrows, as a result of atherosclerotic lesions, restricting blood flow to the heart. Lipid build up in the endothelium is intracellular at first, forming what is known as a fatty streak. As more lipid is taken up a lesion consisting of layers of differentiated macrophages, or foam cells, and lipid droplets develops in the smooth muscle of the artery[83]. This conglomeration of macrophages and accumulated extracellular lipid is accompanied by tissue fibrosis and calcification, as well as inflammation [74]. The narrowing of the arteries may cause angina, or heart pain. Restriction of blood flow can lead to heart failure or plaques may break away from the arterial wall or burst, triggering a heart attack [26].

According to figures from the World Health Organisation, in 2008 around 7.3 million people died from CHD worldwide, accounting for over 12% of global deaths [60]. In the UK, the British Heart Foundation states that in 2007, CHD was responsible for nearly 1 in 5 premature deaths in men and 1 in 10 premature deaths in women [26].

CHD at its most severe can lead to heart failure, and if this gets so advanced that it cannot be managed with drugs then a donor heart may be sought. However, in the vast majority of cases interventions can be performed before this stage is reached. A coronary artery bypass graft procedure may be performed where a donor vein or nearby artery prosthetic vessel graft is used to circumvent the blockage. This requires open heart surgery and although much work has been done in recent years to minimise the trauma associated with this procedure, for example with the increased use of off-pump techniques [62], it remains a major undertaking in terms of risk, recovery period and cost.

Balloon angioplasty is a percutaneous treatment method by which a balloon catheter is delivered, usually via the femoral artery or by the radial artery, to the affected vessel. The balloon is then expanded which compresses the plaque to the vessel wall, restoring the diameter of the lumen. This is far less invasive than bypass surgery and although there is associated balloon injury, the risks are much reduced [50]. However, in 30-50% of cases, the increase in diameter seen immediately following the procedure is not fully retained [54]. This restenosis is attributed to a variable combination of three factors: The influence of the immune system on SMC following compression injury and denudation of the endothelium, vascular remodelling associated with scar contraction and elastic recoil of vessel tissue[99].

A stent is an expandable cage device which can be crimped onto a balloon catheter prior to delivery to the target site. Following expansion of the balloon, the stent is left in place to hold the vessel open, minimising the effects of elastic recoil. Stenting has now become standard practice in percutaneous coronary interventions due to the superior outcomes achieved when stents are deployed versus balloon angioplasty alone. In the influential BENESTENT trial restenosis rates were reduced from around 32% following balloon angioplasty alone to 22% when a stent was deployed as part of the procedure [77].

1.2. Problems in stented vessels

Traditionally stents have been made from medical grade stainless steel (Bare Metal Stents, BMS). They are delivered, expanded at the target site and have been shown to reduce the combined rate of stroke, death and myocardial infarction to a level comparable to coronary bypass surgery[79, 91]. In addition, stenting can be carried out more quickly from the time of diagnosis than surgery,

is significantly less invasive and has notable cost benefits [79].

However, although restenosis is reduced by stenting when compared to rates of 30-50%[54] following angioplasty alone, it is still present in 20-30% of cases[77, 24]. The stent forms a mechanical barrier to the elastic recoil, wound contraction and tissue remodelling which is responsible for around two thirds [99] of late lumen loss following balloon angioplasty. Therefore, although the net result of restenosis in each circumstance is comparable, the mechanisms of in-stent restenosis are not the same; the contribution of neointimal SMC hyperplasia is far more significant [56, 95]. These cells, together with extra-cellular matrix (ECM) molecules, cause a neo-intimal thickening which leads to reocclusion of the vessel, or in-stent restenosis, which in turn restricts blood flow and causes recurrence of symptoms. This means that although restenosis rates are lower than with balloon angioplasty alone, that around 30% of patients will require repeat revascularisation following stenting compared with around 9% following bypass surgery[78].

Vascular SMC exist in one of two main phenotypes; contractile and synthetic, as well as a number of sub-types between these two extremes. In their contractile state, for example in healthy adult blood vessels, SMC demonstrate a low proliferation rate and upregulation of contractile proteins to facilitate healthy vessel function. Conversely, the synthetic phenotype, involved in vascular development, morphogenesis and repair, is characterised by a high proliferation rate and upregulation of ECM production [90]. Phenotypic switching in adult vessels has been implicated in a number of disease states around the body and is particularly important in coronary vessel occlusion by atherosclerotic lesions and restenosis. These changes are thought to result from a combination of mechanical factors and inflammatory mediators [61]. Injury to the vessel and the presentation of a foreign surface to the blood combine to trigger a platelet response to the stent. This activation recruits cells of the innate immune system and subsequent activation of defence mechanisms; Reactive Oxygen Species (ROS) and further pro-inflammatory cytokines [103]. Exposed by the disruption of the endothelium during stent delivery, the SMC of the vessel wall are able to migrate to the vessel lumen [37]. They are also open to infiltration by macrophages which release cell growth factors, further exacerbating SMC proliferation and migration through the injured vessel wall. Restenosis occurring around a given stent strut has been shown to be proportional in vivo to the level of inflammatory cell infiltration [47]. The underlying mechanisms for this are numerous and complex [80]and are likely to involve SMC phenotypic ‘switching’, further discussed in section 1.3. The immune response to the stent surface has also been implicated in restenosis

formation, as some polymers in particular have been shown to induce intense inflammatory responses [89, 49].

In a study published in 2003, Nakatani et al compared the timelines for neointimal growth between balloon injury and stenting in the porcine model. They found that although SMC proliferation in both models began after around 7 days, in the absence of stent struts the SMC reverted to their contractile state after around 2 weeks, compared to up to 28 days following stenting. Morphological changes in the adventitia were observed in the balloon model whereas increased inflammatory presence was observed in the stent model. Taken together, this prompted the authors to speculate that, whereas phenotype switching in the balloon model may be following remodelling signals from the adventitia, the immune response to the stent struts was likely to be triggering the extended period of synthetic activation and consequently the increased neointimal hyperplasia observed following stenting [56]. In terms of the role of mechanical injury, one key influence is likely to be the impact of mechanical stretch on the smooth muscle cells of the medial layer. Indeed Gunn et al (2002) observed that 'Without stretch, there is no neointima'[39]. This may partly be due to the upregulation of protein kinase Akt, a mediator of cell survival and proliferation, shown to occur when SMC are subjected to static stretch as they would be in a stented vessel[105].

Presentation of a foreign surface to the blood not only triggers the immune response leading to restenosis, but it can also in many cases trigger clotting cascades leading to thrombus formation in the vessel. Thrombus formation in the coronary arteries could lead to coronary embolism which can induce myocardial infarction. For this reason it is necessary for patients having recently undergone stent surgery to receive anti-platelet therapy until the endothelium, and thus normal vessel function, has been restored. An increase in vascular complications and length of hospital stay are increased following stenting when compared to balloon angioplasty, however it is considered that these effects are outweighed by the benefits of stenting [77].

1.3. Endothelial cells and vascular healing

Endothelial cells (EC) are important in the synthesis and regulation of antithrombotic molecules as well as providing a surface native to blood flow. Consequently, a healthy endothelium minimises risk of thrombus formation and platelet-induced inflammation. There is a strong argument that the degree to which the endothe-

lium is denudated influences the extent of neointimal hyperplasia [68] and, in general terms, the return of the protective endothelial cell layer to the lumen of an injured blood vessel marks the end-point of vascular healing.

Traditionally the view has been that cells from the uninjured endothelium migrate to cover the damaged region and research indicates that healing can be accelerated in cases where more of the original intima has been preserved[68]. However, there is some uncertainty as to the composition of the lumen surface in an atherosclerotic vessel and whether there would be enough viable EC present to facilitate migration and healing [68]. Indeed, a dysfunctional endothelium may be a factor in the development of some vascular disease, in which case normal vascular healing may not be achievable. More recently, studies have focused on the involvement of circulating CD34+ haematopoietic stem cells, which have the potential for EC differentiation. This has led to a focus on the recruitment of these cells to the stented vessel [43]. However, CD34+ cells are also the progenitor to SMC[102] as well as other cell types from the haematopoetic lineage[11]. Given this heterogeneity and the cocktail of cells and cytokines present in the stented vessel, the true consequences of CD34+ cell recruitment are uncertain. Experimental data using fluorescent labelling techniques in murine models has both confirmed and excluded the incorporation of these cells in the healed endothelium; work by Werner et al and Hagensen et al respectively [97, 40]. Experimental data appears to show that SMC in the neointima do not come from circulating progenitor cells and instead originate from the medial or adventitial components of the vessel wall as discussed in sec. 1.2. However, as with EC this is also under debate [21].

As previously discussed, the change in relatively dormant contractile SMC to the more motile synthetic phenotype plays a key role in SMC hyperplasia and neo-intimal formation. EC are involved in the regulation of SMC phenotype by release of anti-inflammatory or pro-inflammatory factors in response to normal or abnormal shear stresses respectively (Orr et al 2010). Under normal stresses the endothelium produces transforming growth factor beta-1 (TGFß-1) which suppresses differentiation by upregulation of N-cadherin [58]. Conversely, down-regulation of N-cadherin facilitates SMC migration [7]. Interestingly, TGFß-1 is also implicated in the phenomenon of endothelial-to-mesenchymal transition, which effectively describes the differentiation of EC to form SMC in embroyonic development, and it is thought that this mechanistic overlap may be implicated in some cardiovascular disease [21, 35].

1.4. Challenges in stent design

The stent is primarily designed to perform a mechanical function. It is therefore important that in attempting to mitigate against some of the complicating factors described above, the basic stent features required to fulfill this role are not compromised. Perhaps most important is the ability of the expanded stent to hold its form, both radially and laterally. If the stent does not have the radial strength required then it will simply not be able to hold the vessel open. This is made more challenging when you consider the structure must also be closely mounted on the delivery catheter to ensure safe passage through the tortuous route to the target site, as well as the need to reduce the surface area exposed to the blood. Laterally it is less important that the stent retains strength, however it must be resistant to change in length in situ. This is a problem which has been exacerbated more recently with the introduction of stents with increased length as if these shorten the net effect on the vessel is more likely to cause serious complications [67, 101]. Commonly stents form a cage like structure, with open or closed cell formation. This refers to the configuration of the stent struts; a closed cell design is one where struts are interlinked so that each one is supported. For example, a closed cell design may resemble a a wire fence as a series of closed wire polygons. In an open cell design some struts are unsupported so may for example consist of tessellated zigzags joined longitudinally at strategic points[53]. Closed cell designs have an increased radial strength but are more susceptible to variation in length as the diameter to which they are expanded increases. In some stent designs hinges are used to compensate for this[25]. Open cell designs are more accommodating in terms of flexibility, although unsupported struts can protrude into the vessel intima in some designs[98]. Open cell stents have been introduced for use in children as they can permit radial expansion without impact on length[84], although this may be at the cost of radial strength[1]. Changes in strut and cell design can enable stent designers to vary a range of parameters including compression resistance, strength and flexibility[25].

The elastic modulus is another important mechanical consideration in stent design. If the stent is too rigid it may restrict the natural movement within the vessel wall. If it is too brittle then there is a risk of breakage during delivery or in situ. If it is too malleable then the radial strength may be compromised. It is also important that any material chosen for this purpose can easily be visualised from outside the body, with x-ray and MRI compatibility in particular being very desirable.

As with any foreign material implanted into the body it is crucial to consider how the surface chemical structure of a stent may interact in an in vivo environment. This may be in terms of chemical interactions with the material surface wear due to forces the material is under causing ionic or particulate matter to be released [51]. If some release of particles or ions is inevitable then appropriate testing must be carried out to ensure this does not present any additional risk of harm to the patient. Some stent materials are designed to biodegrade over time and in these cases it is important to plan the degradation to avoid toxicity and/or embolus formation. In addition to these considerations, any material which may potentially be implanted should undergo extensive testing in terms of blood compatibility and propensity to induce an allergic or inflammatory response.

1.5. Drug Eluting Stents

Drug-Eluting Stents or DES have traditionally been made from metals coated with polymers which, over time, release drugs designed to reduce restenosis and therefore late luminal loss. The first DES to be developed used Sirolimus, a bacterially produced macrolide antibiotic initially developed as an anti-fungal agent [29]. Previously known as Rapamycin, Sirolimus interferes with the progression of the cell cycle between the first growth phase (G1) and the synthesis (S) phase by interrupting signal transduction pathways. This cytostatic activity was harnessed for use as an immunosuppressant in transplant patients as it inhibits the lymphocyte proliferation associated with rejection [75]. However, following demonstration that antiproliferative effects extend to vascular smooth muscle cells, work began on finding a way to deliver Sirolimus to sites at risk of neointimal hyperplasia. Oral and balloon-catheter administration did not deliver adequate drug concentrations to the target site and so the first DES were developed. The Cypher Sirolimus Eluting Stent (SES) was the first of these devices to be launched commercially. Initial evaluations at 1 year showed a dramatic reduction in need for repeat intervention with the Cypher stent when compared with standard BMS[29]. Most striking is perhaps the RAVEL study, published in 2002 in the New England Journal of Medicine, which demonstrated that while 26% of patients receiving BMS had >50% stenosis at 12 months, this was true for 0% of those receiving the Cypher stent [55].

Around 1 year after the regulatory approval of the use of Sirolimus-loaded DES, another drug was also approved for use in this context. This was Paclitaxel,

eluted by the Taxus stent from Boston Scientific. Paclitaxel is an anti-tumour therapy which inhibits progression of the cell cycle via a mechanism of microtubule stabilisation. At lower doses the cell cycle is interrupted between G1 and S, as with Sirolimus; if the dose is raised then mitotic arrest is induced. Initial reports suggested a comparable level of efficacy between Sirolimus and Paclitaxel with regard to reducing neointimal hyperplasia [29].

Initial reports on DES all appeared to be promising, with an evident reduction in restenosis. However, as their use became more widespread and transferred from clinical studies to 'real world' patients, some negative outcomes began to emerge. Reports began to appear in the literature of an increased rate of mortality amongst patients receiving DES, attributed in particular to late-stent thrombosis (thrombotic events occurring >30 days post –stenting) resulting in fatal cardiac events [29, 44, 92]. Upon autopsy it was seen that in many of these cases although restenosis was at a minimum, neointimal formation was not only reduced in terms of SMC hyperplasia, it was actually incomplete leaving parts of the stent surface bare[44]. In addition, a hypersensitivity reaction, characterised by eosinophil infiltration, had been mounted against components of the implanted stent. Eosinophils are the flagship cell for the immune defence against parasitic invasion, also called the foreign body, or type 2, response. These incompletely healed stented lesions had presented a lethal combination of thrombotic risk, combining bare surfaces with accumulated fibrinous deposits and exposed lipids from the diseased vessel wall [23]. In 2004, Virmani et al reported this hypersensitivity reaction and stated a clear link between this and incomplete neointimal coverage [92]. Attempts were made to try to plot the time course of this process and results from ultrasound measurements previously taken to show that restenosis was not occurring were re-evaluated with the view that in fact these images may be showing delayed vessel healing. It had been known for patients to present with rashes, hives or other allergy-associated symptoms and it was hoped this may be used as a clinical indicator, but it became clear that in many cases effects were so localised that patients were outwardly asymptomatic.

It was thought generally that although when late-stent thrombosis occurred it was serious, indeed 45% of cases were fatal, it was not possible to identify a risk factor individually responsible, or any clinical indicator as to who may be affected. However, evaluation of a cohort of patients who have suffered fatal events cannot constitute full assessment as, with the exception of those who die from other causes, the healing of stented lesions in surviving patients will not be considered. This means it is difficult to assess the risk in terms of the DES population as a

whole[13]. In addition, many fatalities reported were in patients for whom indications for stent surgery were not in accordance with guidelines[100], for example following acute myocardial infarction, or in more complex situations and with longer stents than those previously seen with BMS. DES were therefore not withdrawn, and instead efforts went towards making them safer, with the generation of second-generation devices using drugs such as Zotarolimus and Everolimus. Derivatives of Sirolimus, these drugs are designed to be compatible with less thrombogenic carrier coatings for example phosphorylcholine. In testing, second generation stents appear to show significant improvements in endothelialisation [29].

In 2006, a presentation given by Swiss scientists Edoardo Camenzind and Alain Nordmann at the World Congress in Cardiology in Barcelona ignited a media storm which catapulted the findings that DES may cause fatalities in some circumstances into the public domain. They also suggested that the problem may be more widespread than previously reported as "The problem is likely to be significantly under-reported, since if people die on the street they don't fulfill the angiographic criteria to be classified as in-stent thrombosis" [9]. Although data which otherwise formed part of a more complex picture was sensationalised by the media, and although it was acknowledged that this may not apply to all groups of patients or to second generation devices [20], the media spotlight on the issue meant that research interest expanded and so too the pressure to develop a device which could achieve reduction in restenosis without the portentous risks.

The NHS still prescribe Paclitaxel and '–limus' DES in their less allergenic form. Anti-platelet therapy, usually taken by patients following stenting, is extended to allow for the longer time taken for EC to cover DES stent struts[19]. However, late-stent thrombosis is not a phenomenon exclusive to DES as late stent thrombosis has been shown also to occur in BMS[76]. This means the premature cessation of anti-platelet therapy is a risk factor in both DES and BMS, and there are several studies ongoing to try to optomise treatment times[46]. The most common regime is duel antiplatelet therapy of aspirin together with clopidogrel. Clopidogrel is an anticoagulant which irreversibly inhibits low-affinity ADP receptors preventing platelet activation and therefore the upregulation of membrane glycoprotein receptors necessary for aggregation[73]. The difficulty with long term continuation of drugs such as clopidogrel is the increased risk of bleeding. Side effects such as nosebleeds and bruising can be highly inconvenient for patients over a long period of time, but there can also be more serious effects if other factors are introduced, for instance if a trauma patient is anti-

coagulated at the time of injury. In head-trauma patients there is an increased risk of intracranial complications if the patient has been taking clopidogrel[45]. Additionally there is a significant cohort of patients who are resistant or who do not respond to clopidogrel. Therefore it is necessary to investigate how delayed re-endothelialisation may be addressed in a manner which does not require long term anti-coagulation.

1.6. Current and proposed solutions

A variety of solutions have been proposed to try and resolve the balance between reduction in restenosis and encouragement of endothelial cell growth. As it is likely that drug polymer characteristics form part of the concern in terms of inducing a hypersensitivity reaction, there have been investigations into the material the stent is made from, including polymers and metal alloys. However, as discussed in sec. 1.4 there are many design considerations to take into account as well as potential for systemic toxicity. Attention has therefore been drawn towards the nature of the stent coating, to try and improve biocompatibility.

Newer variations on the DES concept have fallen broadly into three categories: permanent polymer coatings, bioabsorbable polymer coatings and polymer free or minimising designs. Additionally some groups are developing coatings which do not release a drug, or those which may sequentially release more than one drug[30]. Durable polymer coatings build on the lessons learned through first and second generation DES; how they have been effective and where their problems have occurred [49]. Within nanotechnology, new drug-polymer combinations are being tailored to be more tissue-specific and so drug release profiles can follow the cellular chronology in complex lesions[71]. New drugs are also being considered, such as those designed to specifically target neutrophils. As these cells are critical in the first stages of the innate immune response, this is a strategy aimed at minimising rejection before it has truly begun and initial trials appear positive[82].

Some polymer coatings are designed to biodegrade as a loaded drug is released, to be absorbed by the body, leaving a BMS in situ as the surface for EC growth. This concept aims to address restenosis through drug release but without leaving a permanent potentially aggravating polymer surface. The important factors in this design are ensuring firstly that the products of coating breakdown do not cause local or systemic toxicity, and secondly to plan degradation to ensure that as the coating dissolves it does not begin to break apart, as embolus formation

may have serious consequences. The drug elution profile and how it relates to polymer degradation must also be considered. A number of stents with bioabsorbable coatings are currently on the market or in development in Europe. Many of these are sirolimus based and use biologically based polymers, such as poly-L-lactide or polylactic acid, which break down predictably and harmlessly. The ISAR-TEST-4 trial compared biodegradable and permanent polymer stents both eluting the same drug and showed that the biodegradable-coated stent was non-inferior after one year and given the improved long term safety of BMS this is a very promising result [8]. Biolimus A9 is a sirolimus analogue which is very lipophilic meaning that the drugs can cross cell membranes easily. Biolimus A9 was developed by Biosensors, a Swiss company who combine this drug with abluminal polymers in the Biomatrix stent, using these features to minimise systemic exposure by targeting release to local tissues. An additional advantage of abluminal application is that there is no surface interface between the drug coating and the balloon catheter. The Nobori stent by Terumo, linked with Biosensors, follows a similar principle [30].

The Biosensors BioFreedom stent uses Biolimus A9 sprayed directly onto a stainless steel stent. Without a polymer, the drug acts very quickly, peaking within 2 hours, and declining rapidly [86]. Other companies have also tried applying drugs directly without use of a polymer in a form which coats the metal frame and erodes gradually over a period of weeks or months, or also using small reservoirs of drug-polymer to minimise tissue exposure to the polymer surface. Misgivings about the necessarily porous surface this presented to tissues seem to have been dispelled as initial trials have been promising [30].

There have been attempts to coat stents with bioactive molecules such as heparin [85], or to embed them in a polymer matrix and some of these have appeared to show initial success. Stents pre-seeded with EC have also been tried; this idea was less successful due to the impact on the cells of the mechanical stresses of catheter delivery [30]. The Genous EC progenitor capture stent is coated with murine anti-human CD34 monoclonal antibodies. This is designed to capture haematopoetic stem cells with a view to achieving accelerated cell coverage. Initial studies indicated that use of these stents was both feasible and safe [3, 12]. However, an increased SMC proliferation has also been seen which may be due to the potential for CD34+ cells to differentiate into SMC as discussed insec. 1.3. Additionally, as previously mentioned, a dysfunctional endothelium may be a factor in some cases of vascular disease, in which case an approach like this may not necessarily be effective, and it is possible that this is the reason why there have been reports of

late-stent thrombosis in some patients trialing the Genous stent [69, 70, 96]. The area of EC 'fishing' remains a very interesting area of research and other molecules are currently being investigated which may be more specific, an example of which is vascular-endothelial cadherin, expressed only by endothelial progenitor cells.

Research into the mechanical aspects of stent design is also ongoing. There is evidence to suggest that in endothelialised vessels, denudation of the endothelium is as much associated with balloon injury as with stent expansion and consequently self-expanding stents which do not require balloon expansion have been tried[18]. However, these designs have proved to be more difficult to position accurately over the target lesion. Another mechanical innovation was seen in the Xtent which comprised a series of interlocking units which could be separated in vivo to cover longer target lesions without the need for overlapping regions such as those seen when multiple stents are used, and which can often be a focal point for in-stent complications[65]. This design is no longer in development but the problems it aimed to address remain valid. Lesions frequently occur at vessel bifurcations and there are now stents which are specifically designed to cover this area[30]. Indeed, as stents become more commonplace, the lesions they are used to treat are becoming more complex and far away from the indications for the first BMS[36]. Technology is evolving but so too is the spectrum of scenarios in which they may be used and as long as this continues there will always be a need to develop and improve stent designs.

1.7. Conducting polymers

The Nobel prize for chemistry was jointly awarded in 2000 to Alan J. Heeger, Alan G. MacDiarmid and Hideki Shirakawa "for the discovery and development of conductive polymers" for work carried out in 1977 [57]. These are organic polymers which when doped with an electrolyte demonstrate the intrinsic ability to conduct electricity, through a characteristic conjugated structure of alternate double bonds, meaning electrons can be passed along as they would in metallic conduction. These polymers are semi-conductive, though conductivity may vary depending on synthesis conditions, electrolytes and incorporated molecules. This conductivity is comparable to that of inorganic semi-conductors. The production benefits of polymer technology in terms of cost, simplicity and the potential for customisation mean that semi-conductive materials can be tailored for purpose. This technology has found use in many aspects of technological development particularly concerning microelectronics.

Importantly, it has been observed that several conducting polymers have been shown to be biocompatible, leading them to be considered for use in a wide range of medical applications[38]. They have been particularly important in the development of biosensors which require integration between biological sensing molecules and electronic transducers. Other uses have been for tissue engineering scaffolds and implantable electrodes. Each of these applications utilises a different combination of conducting polymer attributes, for example increased conductivity or suitability as a cell growth substrate [38]. In the wake of the initial difficulties with the reendothelialisation of polymer coated DES and following reports of hypersensitivity reactions to polymer implants, research emphasis steered away from utilising polymer substrates in next generation stents. However, recently interest has developed in the use of conducting polymers. This is partly due to their biocompatability, but also the possibility of the incorporation of a wide range of molecules as the polymer is synthesised [4]. This has applications ranging from modifications to the polymer itself, drug loading for subsequent release, or even the loading of whole cells, for example erythrocytes[38]. This means firstly that conducting polymers have potential for use as a drug delivery system but also that adaptations can be made to further customise the device to both environmental and drug-delivery specifications.

1.8. Polypyrrole

In this project, Polypyrrole (Ppy), one of the most widely researched conducting polymers for biomedical applications, is to be explored as a potential growth substrate for EC. In different contexts Ppy has been shown to support growth of many cell types, including neurons which are notoriously challenging to grow on artificial substrates. Wang et al., who examined Ppy as a growth support for peripheral nerve tissue demonstrated that Ppy powder does not cause toxicity or subacute toxicity and is not allergenic, mutagenic, pyrogenic or haemolytic. Their test conclusions were in accordance with international standards ISO 10993 and ASTM F1748-82 [93].

As seen in figure 1, polypyrrole is a chain of five-membered cyclic amine pyrrole monomers which polymerise at positions 2 and 5. The conjugated structure means that the chains are stable and in their standard form carry a net positive charge, enabling the association of dopant anions. In addition, as shown in image 2 in figure 1, R group substitution is possible onto the amino group. The nature

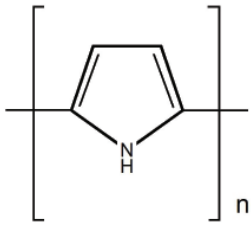


Figure 1.1.: Polypyrrole

of this substitution can allow variation in the polymer, for example a carboxylic acid group would introduce a negative charge or alkyl chain groups may increase hydrophobicity allowing for greater retention of hydrophobic drugs [59]. This adaptability is particularly attractive when considering implantation into highly specialised biological environments. Another positive attribute of Ppy is that it has been shown in some circumstances to act as an anti-oxidant[34], meaning it may itself demonstrate anti-inflammatory activity [64].

One downside is that Ppy can be relatively brittle, meaning much work has been carried out on coated fabrics which provide a greater degree of flexibility [104]. In use of Ppy in a vascular context, sensitive to any jagged surface and particularly embolus formation, this would be important to ascertain that the brittle nature of Ppy did not provide an unfavourable degradation profile. This aspect also becomes relevant when considering the mechanisms of stent expansion and its effect on coating integrity.

1.9. Electropolymerisation of Polypyrrole

Electrosynthesis of Ppy or "pyrrole black", was first performed in 1968[14]. The methods by which this can be done have evolved since then to a system using working, counter and reference electrodes, in a monomer solution with an electrolyte to act as a dopant. Monomers are oxidised at the working electrode, making them highly reactive and inducing the formation of insoluble chains. Here, we propose to generate Ppy on medical grade stainless steel plates using an electrosynthetic approach first utilised in this context by Arbizzani et al in 2007. The nature of this technique means that dopant and molecules to be incorporated are included at the time of synthesis, and the amount taken up can be modulated by charge [4].

The attributes of any Ppy structure grown by electropolymerisation depend on a

number of variables including the structure of the surface on which it is deposited, electrolytes and electrodes used and subsequent reaction kinetics, monomer concentration, dopant used, temperature and pH. It also depends on the voltage applied to the system[5]. One benefit in conducting polymer synthesis, unlike conventional electropolymerisation, is that the growth of the polymer is not limited by its own insulating properties. This means that the thickness of the drug-polymer layer is not inherently limited [5]. In addition, electropolymerisation allows for much finer deposition than chemical synthesis methods and so the combination of these two factors means a high level of control can be exerted on the final thickness of the polymer. Previous work in this department has demonstrated that Ppy surface topography can be varied using voltage modulation through the deposition process and this technique will be utilised here.

1.10. Salicylate

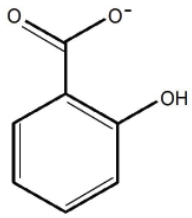


Figure 1.2.: Salicylate

Salicylates are the salts of salicylic acid (figure 2), which forms the basis of acetyl salicylic acid, more commonly known as aspirin. Methyl salicylate exists naturally as oil of wintergreen. Salicylates are non-steroidal anti-inflammatory drugs (NSAID) and, as aspirin, form one half of standard dual anti-platelet therapy following coronary stenting. This means that by using salicylate as a dopant ion no new variables are introduced beyond influence on the nature of the ppy film formed; any impact salicylate may have on EC migration and proliferation would already be present in dual anti-platelet therapy. Additionally, not only is salicylate it an anticoagulant and an anti-inflammatory agent but it has also been shown to impede the proliferation of smooth muscle cells[52]. These attributes make salicylate attractive for treatment of vascular disease and it has been tested previously for use in DES[30]. However, there are reports that aspirin itself, although beneficial as an anticoagulant and anti-inflammatory agent, can itself inhibit EC growth [66]. Sodium salicylate (NaSa) has been shown to work well

as a dopant for electropolymerisation of ppy[4] and has been researched in an industrial capacity as an anti-corrosion technique[2]. On a zinc substrate it was found that interaction with the metal ions on the surface enabled it to form a particularly stable coating[41][81].

1.11. Impact of surface characteristics on reendothelialisation

The surface characteristics of implanted stents or vascular grafts have a significant impact on reendothelialisation[51]. In many cases this is due to the ease with which plasma proteins can adsorb to the surface in order to provide support for focal adhesion. The adsorption of proteins is reliant on the charge of the surface exposed to blood plasma and the charge at the material surface may mean that proteins attach in an unfavourable conformation. Additionally, if the wettability of the surface is too great then water molecules will form a barrier to strong adhesionBacakova et al. [6]. The nature of the bonds formed at attachment is also important; whether proteins attach covalently, electrostatically, or by hydrogen or ionic bonds. It has been shown that molecules can be covalently attached to some Ppy surfaces[42]. This may be advantageous in maintaining robust attachment in the coronary vascular environment where EC are under shear stresses of between 0.33 and 1.24Pa [16]. Covalent bonding between ECM molecules and an underlying substrate has been shown to be important in ensuring cells are not washed away[87]. If Ppy has the capacity to bond covalently then with further research it may be possible to maximise this potential. There is evidence to suggest that shear forces influence the size and orientation of focal adhesions generated by EC.Teichmann et al. [88]. These focal adhesions would not therefore be represented in a static system.

As this project has a focus on EC and SMC growth then it is the surface topography which is of particular relevance. It has been shown that changes in the topography of a surface can influence the propensity for cells to migrate and proliferate. Again, this is thought in some part to be related to protein adsorption; on rougher surfaces there is a greater surface area presented to the blood and access of ligand sites for cellular adhesion molecules may be improved. Nanostructured surfaces, that is to say those with topographical variance at the nano level, have been shown to be particularly supportive and it is suggested that this may be as they may more closely resemble the naturally occurring ECM. It has

also been suggested that nanostructures, due to their size in relation to plasma proteins such as fibronectin and vitronectin, can exert an influence over which proteins adsorb to the surface of an implanted materialBacakova et al. [6].

When a foreign surface is exposed to blood plasma or tissue fluid, it is immediately covered with a layer of proteins and it is the nature of these proteins which forms the basis of any tissue reaction, be that platelet or immune activation or cell recruitment or migration. It is therefore critical in the design of any material for implantation to consider which proteins may be attracted to the surface in question and in what configuration. Many studies use surfaces enhanced by factors such as fibronectin but in vivo there may be other molecules which bind to a given substrate more prominently[6]. Furthermore, a polymer co-synthesised with particular biologically active molecules may not necessarily ever be in direct contact with the cell it is designed to influence[32].

1.12. Study hypothesis and experimental aims

These experiments monitor the growth of endothelial and smooth muscle cells on a Ppy surface with variation in topography achieved through voltage modulation during electropolymerisation. Ppy synthesised by using sodium salicylate as a dopant ion and eluted drug will be presented to SMC and EC in solution and their responses guaged in terms of confluence, morphology and viability

Here we hypothesise that polymerisation techniques previously developed based on the work of Arbizzani[4] will transfer from the surface of a wire to that of a stainless steel plate. We hypothesise that viable cultures of SMC and EC can be generated on a ppy surface, that variation in topography will influence the proliferation of SMC and EC, and that this influence will differ between cell types. This speculation is derived from remarks in the literature that nanostructures attract a larger percentage of vitronectin, a preferred EC substrate [6]. In addition we hypothesise that EC will have the capacity to migrate on a Ppy surface.

Finally, we hypothesise that loading of salicylate will not significantly impede cell growth when compared with administration of the same drug to the cell suspension, thereby demonstrating no significant adverse effect uniquely attributable to the polymer.

The overall aim of this work is to assess the suitability of polypyrrole for use in the formulation of a novel, EC-supportive durable drug-polymer for stent applications.

2. Methodology

2.1. Introduction

Chapter 1 highlights a potential role for conducting polymers in drug-eluting stent development, suggesting that they may provide surfaces conducive to cell culture and setting an objective of this study to determine the extent to which polypyrrole surfaces can support vascular cells. Here we set out the materials and methods used in addressing this overall objective. The chapter firstly describes the electropolymerisation method used to produce a series of novel polypyrrole coatings. It goes on to outline the conditions for the culture of endothelial and smooth muscle cells (EC and SMC). The chapter concludes with a description of the methods adopted to assess the viability, adhesion and migration of these cells on the novel polypyrrole surfaces produced.

2.2. Polypyrrole Coating Production

2.2.1. Materials

Sodium Salicylate (NaSa) and pyrrole monomer (Py) were purchased from Sigma-Aldrich (Poole, UK). Stainless steel sheets (stainless steel, AISI 316L grade, annealed, 150mm x 150mm x 0.5mm) were purchased from Goodfellow Cambridge Ltd (Huntingdon, UK). These sheets were cut into 20mm x 20mm x 0.5mm sections for coating. The platinum wire counter electrode (1mm diameter) was also purchased from Goodfellow Cambridge Ltd (Huntingdon, UK). A KR5 reference electrode was purchased from ThermoScientific UK Ltd (Leicestershire, UK)

2.2.2. Equipment

Electropolymerisation conditions were controlled using an electrochemical interface (SI 1287, Solartron Analytical, Hampshire, UK)

2.2.3. Method

The electropolymerisation techniques to be used in the present study are based around the protocols used by [4]. Electropolymerisation was carried out at room temperature in aquaeous solutions of 0.1M Py – 0.1M NaSa or 0.1M Py – 0.1M NaCl prepared in deionised water. The polymerisation was performed in a three-electrode cell. A platinum wire (1mm diameter) was used as the counterelectrode and a KR5 electrode acted as the reference. Fig. 2.1 shows an example of a polymer coated foil, and the electrochemical cell used. Formation of the polypyrrole material occurred at the working electrode, which was a 20x20mm square of stainless steel foil. Immediately prior to the coating procedure commencing, the stainless steel electrode was rinsed in ethanol in order to remove any particulate debris.

Electropolymerisation was performed using a Solartron 1287 galvanostat by two methods: potentiostatic and cyclic voltammetry. For potentiostatic synthesis, a constant of 0.9V was used to produce electropolymerisation. For cyclic voltametric synthesis the voltage was cycled a total of five times between 0.5 and 2.0V at a scan rate of 40 mv/s. Voltage levels selected were based on previous levels reported in the literature[4, 59] and to provide consistency with work being carried out in a parallel project within the laboratory. Potentiostatic synthesis of Py-Sa was carried out over a 15 minute period to allow sufficient polymer deposition to take place. As deposition of Py-Cl was less successful and progressed only for a short time, a 5 minute period was used for this synthesis process. Following the electropolymerisation, the stainless steel electrode was removed and left to dry at room temperature overnight prior to use in cell-based assays.

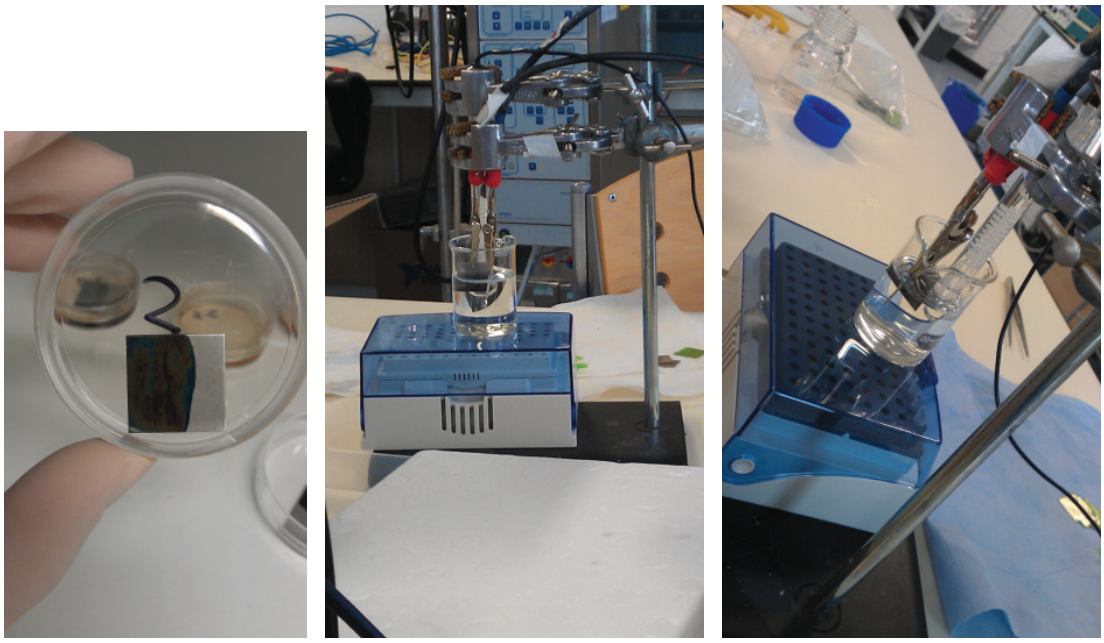


Figure 2.1.: Left: An example of a coated stainless steel foil, as used experimentally.
Centre/Right: The electrochemical cell. Note the fixed nature of the clamping apparatus, meaning the depth of immersion is held approximately constant, assuming consistency is maintained in the volume of coating solution. Angles of the working and counter electrode are adjusted for each trial to optimise polymerisation.

2.3. Cell culture

2.3.1. Materials

TrypLE™ Express dissocation medium, Large vessel Endothelial Cell medium, Low Serum Growth Supplement, Weymouth's MB 752/1 Meium and Ham's F12 Nutrient Mix were all purchased from Life Technologies (Glasgow, UK). Foetal Calf Serum (FCS) was purchased from Sigma-Aldrich (Poole, UK). Fresh pig hearts obtained from John Robertson and Sons (Ham Curers) Ltd. (Ardrossan, Ayrshire, UK). Penicillin and streptomycin acquired from PAA - The Cell Culture Company (Austria)

2.3.2. Equipment

Light microscopy was carried out using a Nikon Diaphot inverted microscope. Centrifugation used a MSE mistral 2000 benchtop centrifuge. All sterile work was carried out in a laminar flow workstation supplied by Bassaire Ltd (UK).

2.3.3. Method

Primary porcine vascular endothelial cells, isolated from the pulmonary artery of pig hearts were cultured in Large Vessel Endothelial Cell medium, with Low Serum Growth supplement and 1% penicillin/streptomycin antibiotic solution. Smooth muscle cells isolated from the left anterior descending coronary artery were cultured in 50:50 Weymouth's MB 752/1 Medium and Ham's F12 Nutrient Mix, with 1% penicillin/streptomycin antibiotic solution and 10% FCS. In both cases the medium was selected to optimise growth of the desired cell type and was based on the results from previous work carried out in the laboratory. Cell media was changed every 2-4 days and cells were passaged when approaching confluence. Cell cultures were confirmed as of the correct type through morphology; cobblestone and hill-and-valley for EC and SMC populations respectively. Previous work within the laboratory has confirmed the specificity of these isolation procedures for the two cell types studied.

Cells were lifted for passage by incubation with 2.5ml or 5ml TrypLE solution for T25 and T75 flasks respectively until the point at which lifting could be observed as complete by light microscopy. The TrypLE was then neutralised with Weymouth's MB 752/1 medium and cell suspensions were then centrifuged

at 200G for 5 minutes, before being resuspended in the relevant culture medium and transferred to a new culture flask. The cell proliferation rate was found to be variable and so a regular passage routine was not established for either cell type. Instead, cells were examined daily using the light microscope and passaged as and when necessary.

2.4. Assessment of cell adhesion, viability and migration on polypyrrole surfaces

2.4.1. Materials

Sodium Salicylate, Acridine Orange, phosphate buffer solution, formalin, pyruvic acid and NADH were all purchased from Sigma-Aldrich (Poole, UK). All cells used were at passage numbers 2-3

2.4.2. Equipment

Fluorescence images were captured using a microscope made by Carl Zeiss (Germany), in conjunction with Axiovision software. Surface images were captured using Hitachi TM100 Scanning Electron Microscope. Experimental cell cultures were carried out using BD Falcon™ 35 mm Easy-Grip™ Cell Culture Dishes and all sterile work was carried out in a laminar flow workstation supplied by Bassaire Ltd (UK). LDH activity was measured using a Shimadzu UV-2401 PC UV-VIS Recording Spectrophotometer.

2.4.3. Method

Throughout this section, samples will be named as follows:
NaCl-P: NaCl dopant ion, synthesised under potentiostatic conditions at 0.9V
NaSa-P: NaSa dopant ion, synthesised under potentiostatic conditions at 0.9V
NaSa-C: NaSa dopant ion, synthesised using cyclic voltometry over 5 cycles between 0.5V and 2.0V

2.4.3.1. Culture preparation

The coated plates produced in sec. 2.2 were sterilised in 70% ethanol and left to dry in a clean room environment. These plates were then placed in 35mm

tissue culture dishes and incubated at 37^{o} for 1 to 2 hours in FCS. FCS is present in standard culture medium so this incubation was designed to amplify surface protein adsorption without introduction of any new factors to the experimental system or bias towards any particular protein. This step has been used for other artificial cell substrates and been shown to be beneficial to cell growth[15, 32].

As salicylate is presumed to be eluted from the surface of some of the Ppy samples, a control sample was included in all experiments containing salicylate only, to isolate any potential effects of the drug on cell responses. For this, Salicylate was present in the culture medium at a concentration of 1 x 10^{-6}M. A solution was made in deionised water of NaSa at 1 x 10^{-4}M, sterilised by filtration then 40µl added to each 4ml dish. 40µl of deionised water was added to all other dishes.

2.4.3.2. Smooth Muscle Cell Seeding - Experiment 1

NaSa-C and NaSa-P ppy samples were tested. Control dishes were established to assess growth on tissue culture plastic only and tissue culture plastic with NaSa in the medium at 1 x 10^{-6} M as described above. NaCl-P samples were also included with and without NaSa in the medium; this was to monitor the influence of Ppy without NaSa present. 6 plates were prepared in duplicate, to be assessed at a 2 day and 5 day time points.

Two T75 flasks of SMC were prepared as described in sec. 2.3.3. These cells were suspended in 8ml of medium. 3ml of medium was added to 35mm tissue culture dishes along with 40µl of NaSa solution or distilled water as appropriate. 1ml of cell suspension was then added to each culture dish to provide a total of approximately 4ml medium, which was sufficient to completely immerse the polypyrrole coated metal sheets.

At 2 and 5 day time points medium was removed and replaced with 10% formalin solution to fix cells. The composition of these 6 plates can be found in Tab. 2.1. Dishes were covered with foil to exclude light and refrigerated pending evaluation. Cells were not counted prior to experimentation however as control plates were made from the same suspension and over the same time points their cell counts are assumed to have been equivalent.

2.4.3.3. Smooth Muscle Cell Seeding - Experiment 2.

This experiment was intended as a repeat of sec. 2.4.3.2, following the same procedure with the hope of replicating the findings of the experiment. However, as

Sample	Polymer	1x10^{-6}M NaSa
1	None	No
2	None	Yes
3	NaSa-P	No
4	NaCl-P	No
5	NaCl-P	Yes
6	NaCl-C	No

Table 2.1.: Experiment 1: 6 cell culture environments prepared, duplicated for assessment at 2 and 5 day timepoints

the growth rate of SMC in culture appeared to have slowed, it was clear that the cell density of each T75 flask was reduced. It was therefore decided that each T75 was therefore only split between 4 dishes to maximise the cell count in each, and that the NaCl-P samples would be excluded. Control wells were again included so that experimental results could be viewed in the context of this cell culture and not that which was used for experiment 1.

2.4.3.4. Endothelial Cell Seeding and Migration

Endothelial cells underwent similar treatment to that previously described for smooth muscle cells insec. 2.4.3.2. Cells were available in T75 flasks at full confluence. As their growth had been observed to be reasonably rapid it was judged that cells should be delivered at approximately two-thirds confluence. The area of a T75 is 7500mm^2 and the area of a 35mm dish is 962mm^2 so the cells from one flask was resuspended in 10ml of medium and 1ml of cell suspension was added to each dish.

Informed by the results of experiments 1 and 2, it was judged that the EC would be delivered to NaSa-P only. 6 dishes were prepared, as per Tab. 2.2. At the 2 day time point cells were removed cleanly from the Ppy and steel surfaces on one side using a sterile cell scraper, shown schematically in Fig. 2.2. The migration observed across the denuded side on the 5 day sample could then be compared to the sharp line between monolayer and scraped surface on the two day sample.

2.4.3.5. Vascular Cell Viability

The LDH assay is a common test to quantify the health of cells in culture, using membrane integrity as a measure of cell survival. If cells die and rupture then enzymes can leak out into the culture medium. LDH is an enzyme present in

Sample	Polymer	$1x10^{-6}$M NaSa
1	None	No
2	None	Yes
3	NaSa-P	No

Table 2.2.: EC experiment: The 3 cell culture environments prepared, duplicated for assessment at 2 and 5 day timepoints

Figure 2.2.: A schematic approximating the area (green) denuded using the cell scraper on the NaSa-P after 2 days

many body tissues which converts pyruvic acid into lactic acid, a process which oxidises NADH. NADH absorbs light at 340nm and so its concentration can be measured spectrophotometrically. If LDH concentrations are high then over a 60 second kinetic analysis a steep linear curve will be displayed as the reaction progresses. If LDH concentrations are low, NADH levels will remain relatively constant.

100µl of sample to be tested was added to a 1ml cuvette with 0.86ml of 0.1M Sodium Phosphate buffer (NaPi buffer) at pH 7.6. To this 40µl of solution containing excess pyruvic acid and NADH was added. This solution is 3mg of each compound mixed in 1ml of NaPi buffer and kept on ice. The cuvette was inverted 3-4 times and samples were analysed immediately for NADH levels over a 60 second period.

Results can be calculated by measuring the change in NADH between 0 and 60 seconds. This change is divided by the molar extinction coefficient, 6.22mM-1. This gives the LDH activity in 100µl in µmol/ml/min. For a dish containing 4ml medium then this would be multiplied by 40 to get the amount of LDH in each dish.

Positive controls were carried out by removing medium from a dish of healthy cells and replacing it with 1% w/v Triton-X-100 solution which causes cells to be killed. Dishes were assessed by light microscopy to ensure cells had been

destroyed and then 100µl of the solution was taken from each dish and analysed as previously described. This method allows the effect of killing the cells to be compared directly as both the original medium and the Triton-X solution from the same dish can be analysed.

2.4.3.6. Vascular Cell Adhesion

Cell adhesion to the polymer surface is judged qualitatively in terms of confluence, morphology and adherence, through fluorescence imaging of fixed samples. These were stained using Acridine Orange which binds to DNA and RNA emitting green and orange fluorescence respectively. Surfaces were carefully washed using PBS at pH 7.4 3 times to remove traces of formalin. They were then immersed in acridine orange stain solution, incubated in darkness for one minute, then rinsed again 3 times in PBS. Imaging was carried out immediately using fluorescence microscopy (Zeiss) in conjunction with Axiovision software. Images were taken which detected green and red fluorescence. To capture the boundaries between steel and polymer images were combined with those taken using Differential Interference Contrast (DIC) microscopy which is a reflective technique designed to capture 3D properties of the viewed sample.

2.4.3.7. Endothelial Cell Migration

Procedurally, assessment of these plates was carried out as in sec. 2.4.3.6. Assessment of migration focused particularly on the boundaries between the monolayer and denuded regions as described in Fig. 2.2.

2.4.4. Surface Characterisation

Polymer surfaces were analysed with and without cell growth using a Hitachi TM100 Scanning Electron Microscope .

3. Results

These experiments aimed to monitor the proliferation of endothelial cells on a Ppy surface with variation in topography achieved through voltage modulation during electropolymerisation. Ppy synthesised by using sodium salicylate as a dopant ion and eluted drug were presented to SMC and EC in solution and their responses gauged in terms of confluence, morphology and viability

Throughout this section, samples will be named as follows:
NaCl-P: NaCl dopant ion, synthesised under potentiostatic conditions at 0.9V
NaSa-P: NaSa dopant ion, synthesised under potentiostatic conditions at 0.9V
NaSa-C: NaSa dopant ion, synthesised using cyclic voltometry over 5 cycles between 0.5V and 2.0V

3.1. Polypyrrole production

Fig. 3.1 and Fig. 3.2 show results of polypyrrole synthesis carried out using sodium salicylate and sodium chloride dopant ions respectively. The difference in the quality of coatings is clearly visible. Films using salicylate dopant ions formed readily and appear visually to be even, whereas those using chloride ions were less predictable, were visually less consistent and at some points the metal appears to have been oxidised. The following sections go on to demonstrate the results of cell culture on these surfaces.

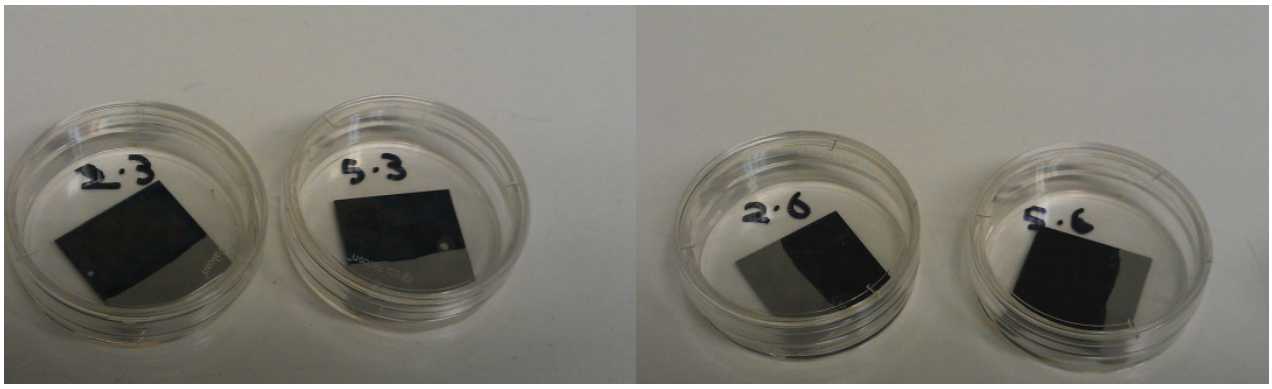


Figure 3.1.: Surfaces synthesised using NaSa dopant ions. (Used in experiments shown inFig. 3.7 and Fig. 3.8:- *2.3*: 2 day, NaSa-P. *5.3*: 5 day, NaSa-P. *2.6*: 2 day, NaSa-C. *5.6*: 5 day, NaSa-C)

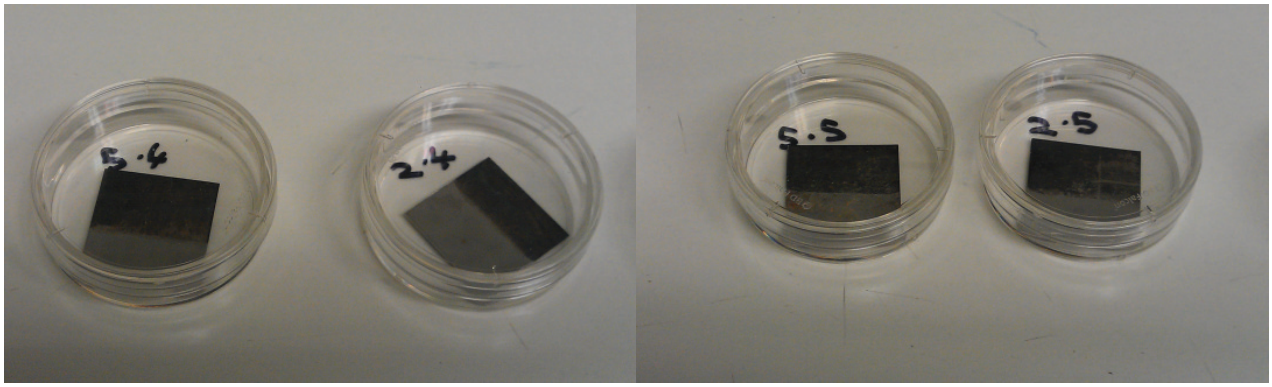
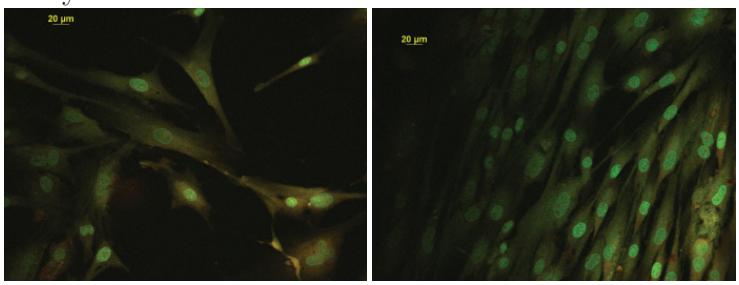


Figure 3.2.: NaCl-P surfaces prior to sterilisation in alcohol. Note the poor quality of coating, particularly on surface 2.5, as well as signs of steel oxidation on surface 5.5. (Used in experiments shown inFig. 3.5:- *5.4*: 5 day, no NaSa. *2.4*: 2 day, no NaSa. *5.5*: 5 day, plus NaSa. *2.5*: 2 day, plus NaSa)

3.2. Experiments 1 and 2: Smooth Muscle Cells.

In order to determine the extent to which SMC would successfully adhere to the polypyrrole surfaces created in sec. 3.1, SMC were seeded onto these surfaces and their adherence was assessed at two and five day time points. The results of this work are summarised in figures (Fig. 3.5,Fig. 3.7 and) and discussed below.

2 day



5 day

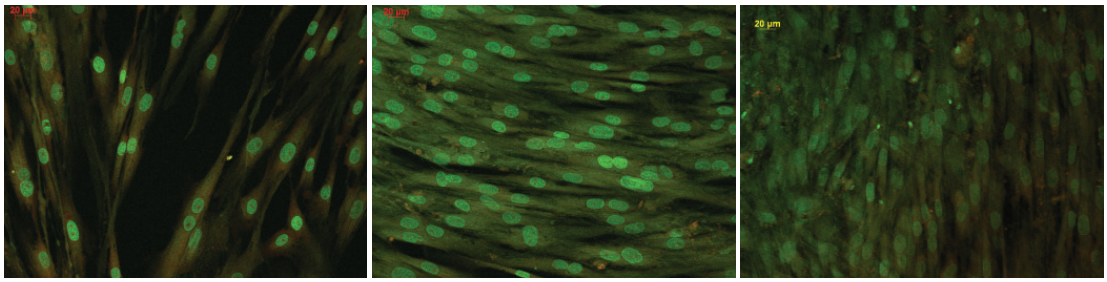
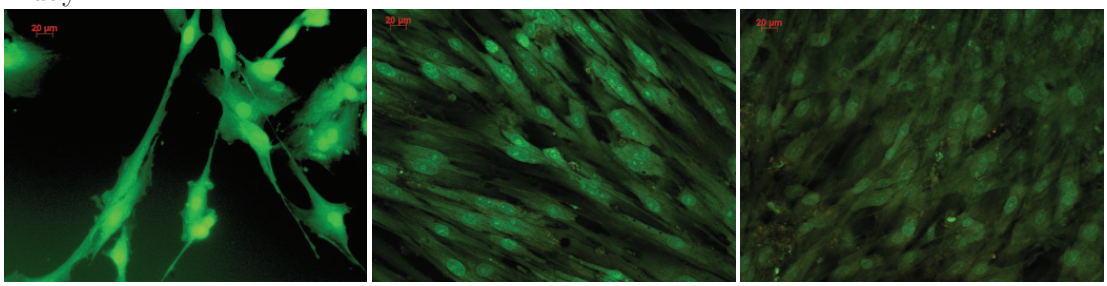


Figure 3.3.: Control proliferation of SMC under normal conditions

Fig. 3.3 shows the progression of SMC population growth on tissue culture plastic, at two days and again at five days. It can be seen that cells are following the hill-and-valley morphology characteristic of this cell type. At two days samples were found to have cell-free regions of the plastic. However in those samples observed at five days the extent of cell-free space appeared greatly reduced. This control indicates normal cell proliferation.

2 day



5 day

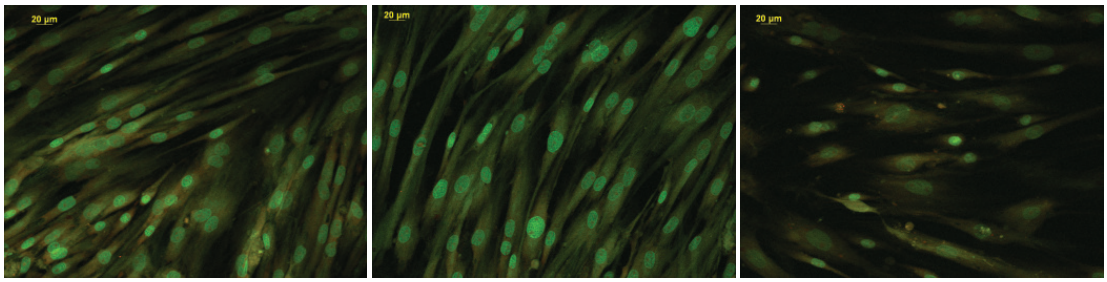


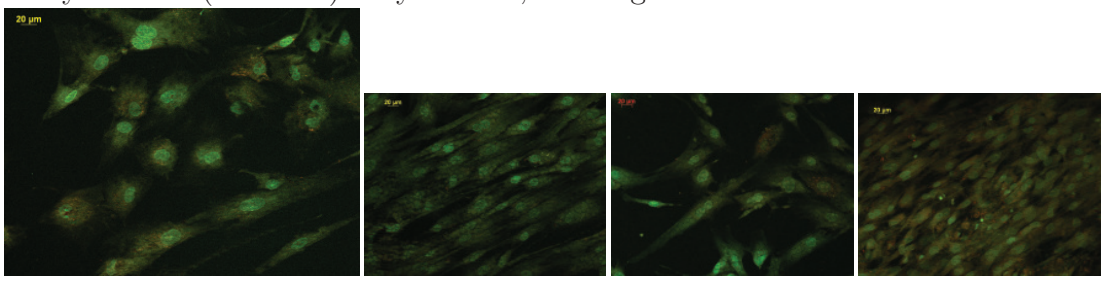
Figure 3.4.: Control proliferation in medium containing NaSa at $1x10^{-6}$ M

Fig. 3.4 shows the progression of the same SMC cells at the same time points as figure 1, cultured in growth medium containing NaSa at $1x10^{-6}$M. The presence of the drug does not appear to have prevented cell survival and proliferation, however the growth rate appears reduced, as evidenced by low-cell regions of the plastic after 5 days, something which was only observed at the far edges of the control sample. Additionally a greater variation in morphology may be observed.

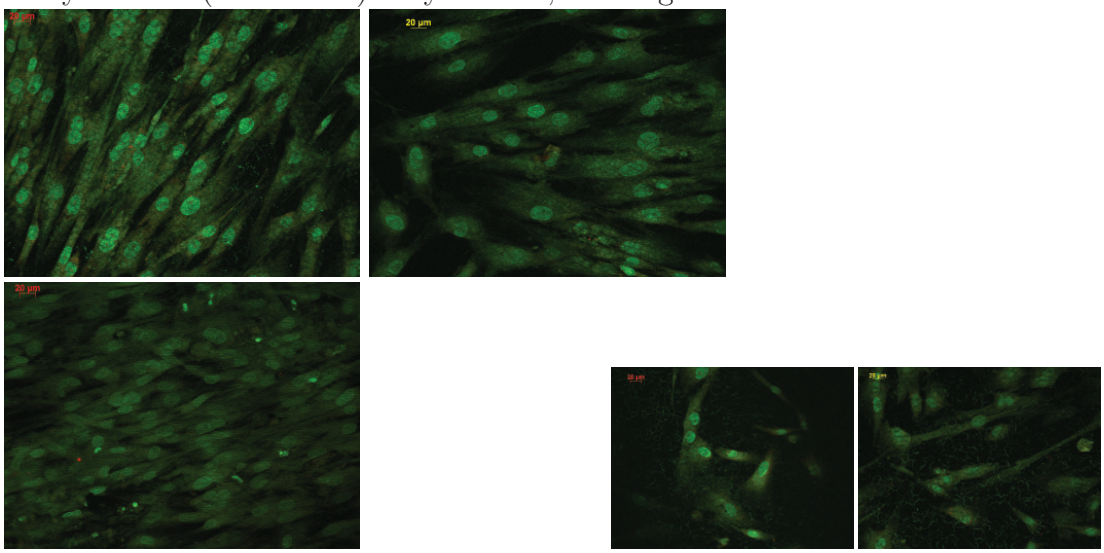
Fig. 3.5 shows results from control surfaces made using NaCl dopant ions. Encouragingly one of the ppy surfaces attracted a level of proliferation considerably stronger than any other, including the metal and tissue culture plastic. This was despite presence of NaSa in the growth medium. However results were very inconsisitent and as the images show, only one of the samples attracted this level of cell adherence and even the same conditions at 5 days did not achieve the same cell response. Even on each individual surface a high level of variability was seen, consistent with the poor quality of the coatings achieved(Fig. 3.2). Under bright light (DIC) it was possible to visualise scratches on the metal surface of one of the samples (Fig. 3.6) and, as mentioned previously, the steel in some parts appears also to have oxidised in places during the electropolymerisation process which may also have contributed(Fig. 3.2).

Fig. 3.7 shows results from NaSa-P surfaces. At two days adherence on the polymer is comparable to that on stainless steel. At five days there are a large number of cells on both the metal and the polymer. However the morphology is more consistent in cells on the metal surface. Some clumping of cells can be observed on the polymer surface after 5 days.

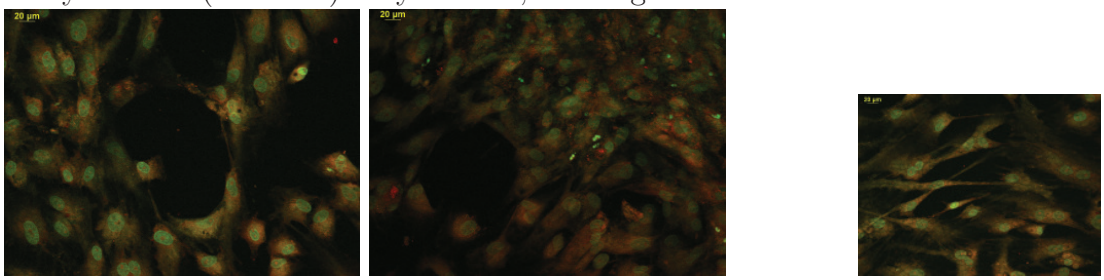
2 day NaCl-P (no NaSa) Polymer left, steel right



2 day NaCl-P (with NaSa) Polymer left, steel right



5 day NaCl-P (no NaSa) Polymer left, steel right



5 day NaCl-P (with NaSa) Polymer left, steel right

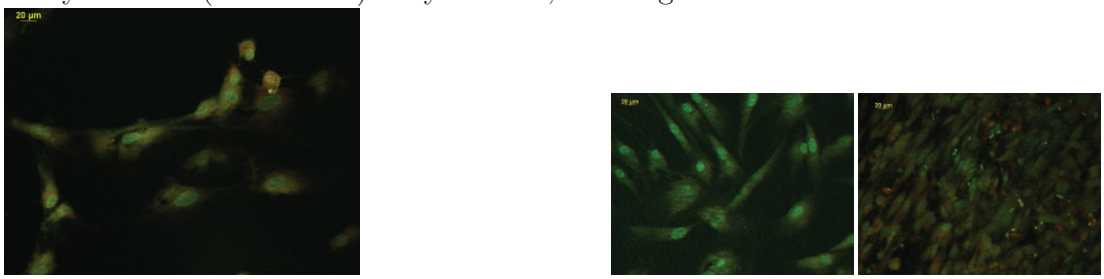


Figure 3.5.: Cell adherence on control NaCl-P and the respective uncoated stainless steel section for each polymer coating. Multiple images are included to reflect variation whereas single images indicate an observation is typical. From the top: Cell adherence at 2 days on NaCl-P *(left)* and steel *(right)*. Adherence in medium containing $1x10^{-6}$M NaSa at 2 days on NaCl-P *(left)* and steel *(right)*. Cell adherence at 5 days on NaCl-P *(left)* and steel *(right)*. Adherence in medium containing $1x10^{-6}$M NaSa at 5 days on NaCl-P *(left)* and steel *(right)*.

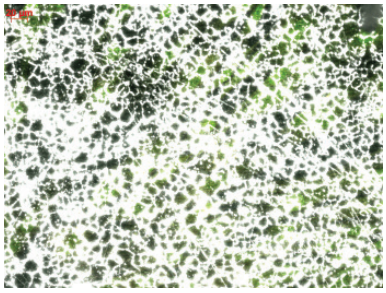


Figure 3.6.: Scratches on the metal surface in the crossover region on sample 2.4 *(NaCl-P for the two day time point with no NaSa added)*

i.

ii.

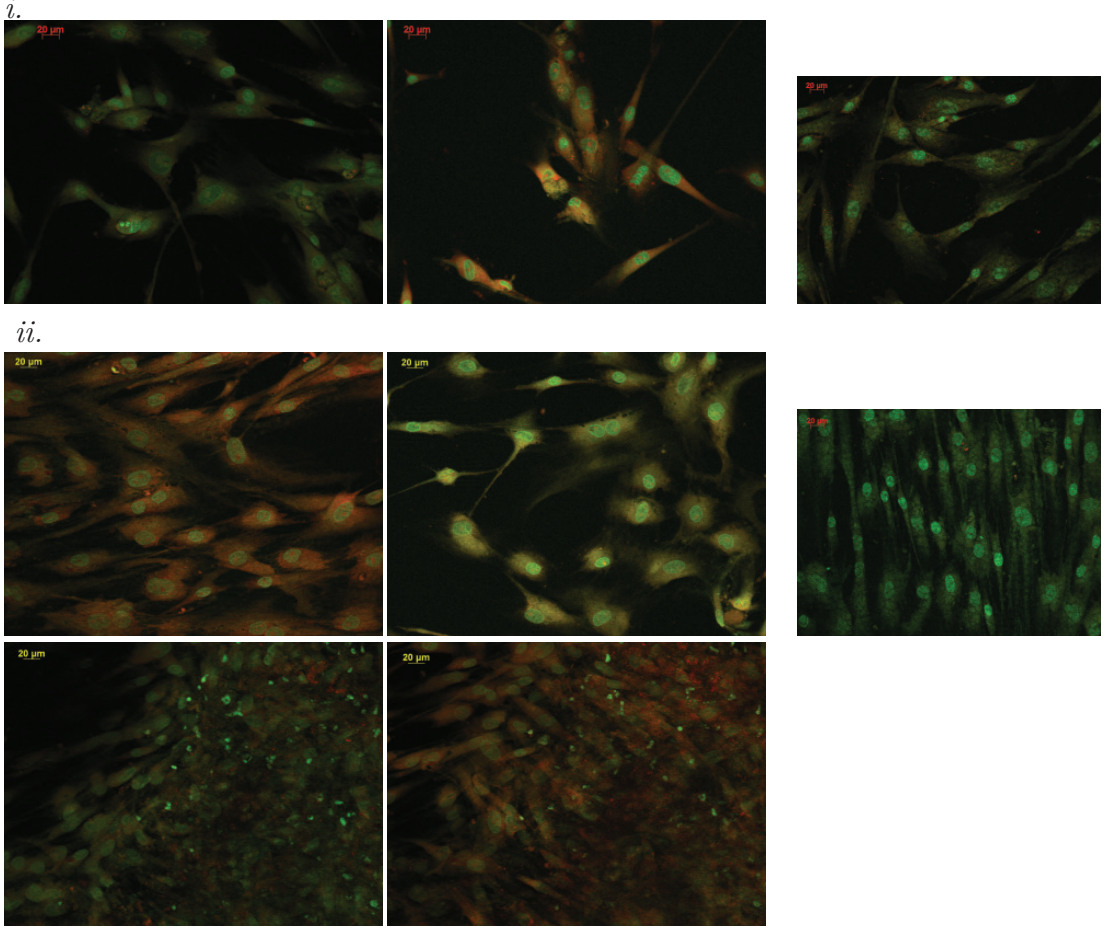


Figure 3.7.: Cell growth on NaSa-P ppy surfaces and uncoated stainless steel. Multiple images are included to reflect variation whereas single images indicate an observation is typical.
i. adherence at 2 days on ppy (left) and steel (right). *ii.* adherence at 5 days on ppy (left) and steel (right).

In comparison with NaSa-P, NaSa-C surfaces show an increased variability in cell adherence at both two and five day time points(Fig. 3.8). This includes the clumping effect seen previously. As in Fig. 3.5, no regions on the metal can compare with the strongest areas of proliferation on the polymer. Conversely however, there were also no areas on the metal which were sparsely populated as the weakest areas on the polymer.

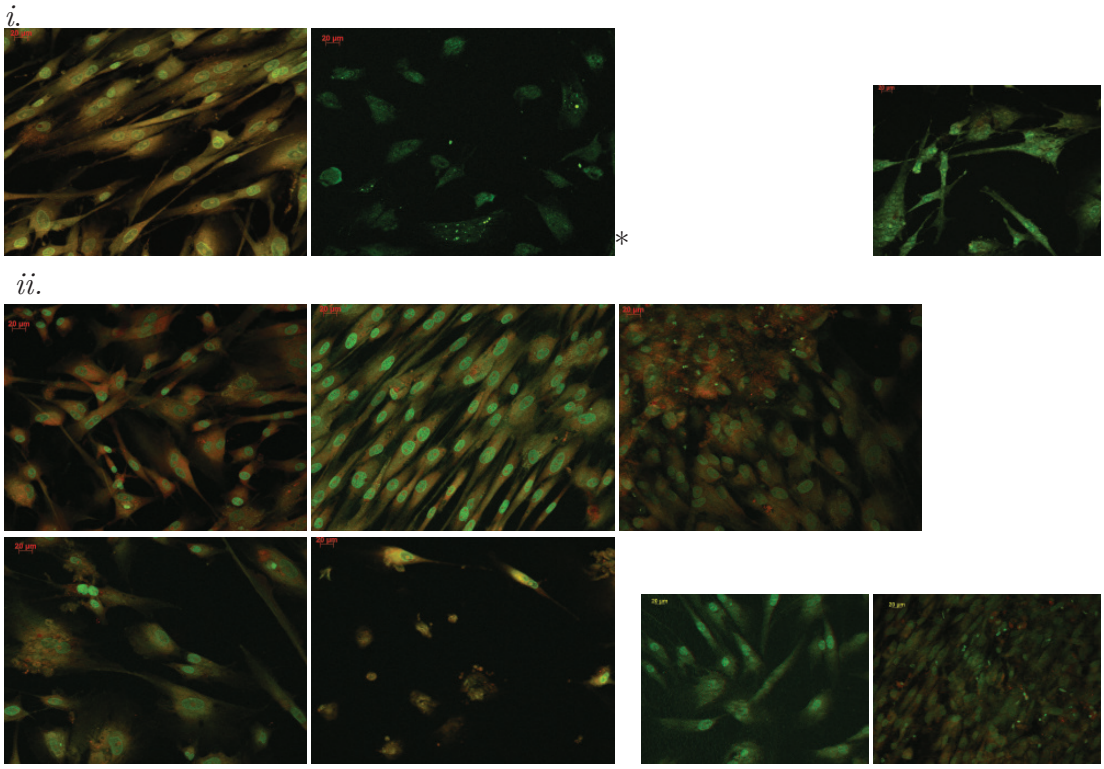


Figure 3.8.: Cell adherence on NaSa-C ppy surfaces and uncoated stainless steel. Multiple images are included to reflect variation whereas single images indicate an observation is typical.

i. adherence at 2 days on ppy (left) and steel (right). *ii.* adherence at 5 days on ppy (left) and steel (right)

*image contrast has been adjusted for visibility. See original in Appendix 2

LDH assays are a quantification of cell survival in terms of enzyme leaked as a result of loss of cell membrane integrity. Elevated levels of enzyme activity indicate a high incidence of cell death. Fig. 3.9 shows the difference between minimum and maximum LDH values which can be obtained. The positive and negative control values shown are from the same cell cultures before and after treatment with Triton-X which induces cell necrosis. As can be seen inFig. 3.10, in experiment 1 the viability of cultures containing polypyrrole is comparable to that seen in the control where cells are grown under standard conditions.

There is a spike of LDH activity for the NaSa-C polymer. That some cells may not survive on these surfaces appears consistent with the levels of variability observed by microscopy(Fig. 3.8). Images for the 2 day timepoint are n=2 as they also include data from medium from the 5 day experiment undergoing a medium change. As only these cells continued to 5 days, the LDH assay at the later timepoint can only be n=1.

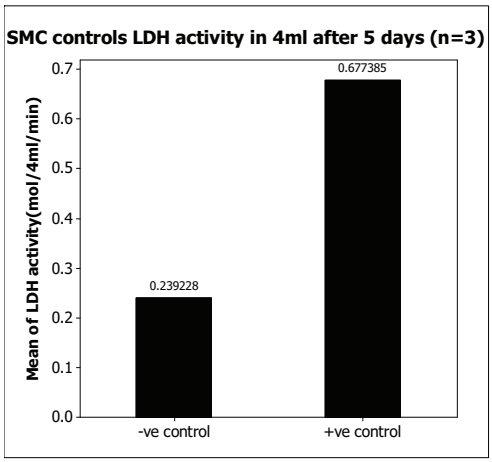


Figure 3.9.: Control Samples of LDH assay using SMC

Negative control: triplicate samples grown over 2 days, tested for LDH release, then killed using Triton-X to establish a maximum LDH level (*positive control*)

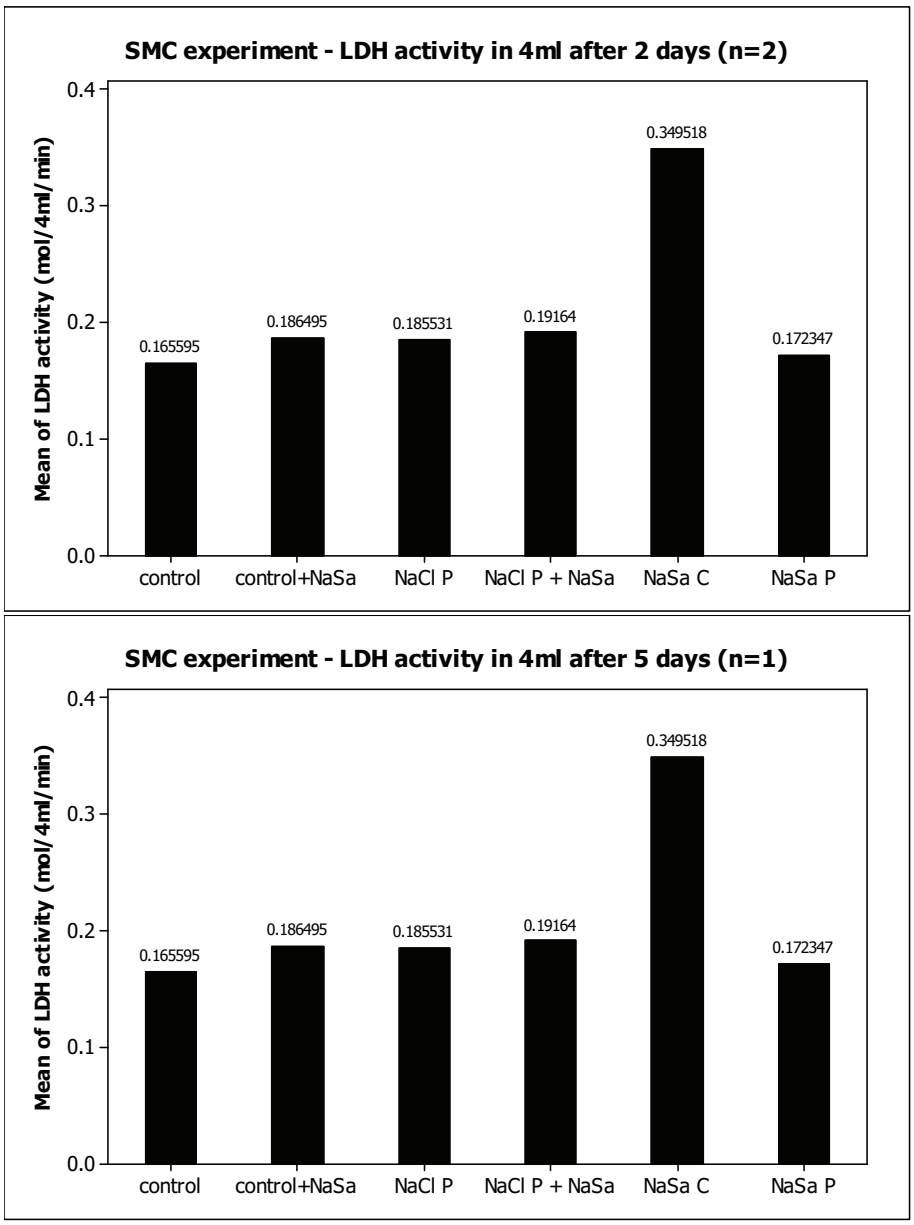


Figure 3.10.: Graphs showing levels of LDH activity in terms of NADH used, in medium taken from cell cultures used in Experiment 1 at 2 and 5 day time-points. . Samples left to right:- *Control*: cells used experimentally grown under standard conditions. *Control + NaSa*: including NaSa solution at 1 x 10^{-6}. *NaSa-P*. *NaCl-P*. *NaCl P + NaSa*: including NaSa solution at 1 x 10^{-6}. *NaSa-C*

As mentioned in sec. 2.4.3.3, the number of samples tested in experiment 2 was reduced due to poor cell growth rates. Unfortunately, this continued in the experimental samples, as shown in Fig. 3.11, meaning it could not reasonably be considered a repeat of experiment 1. Fig. 3.12 shows LDH data for experiment 2. All samples show relatively low levels of LDH however with reduced proliferation it is difficult to know if this can be attributed to cell viability or low cell numbers. Additionally the relevance of the control data is called into question as these were obtained using cells growing more successfully.

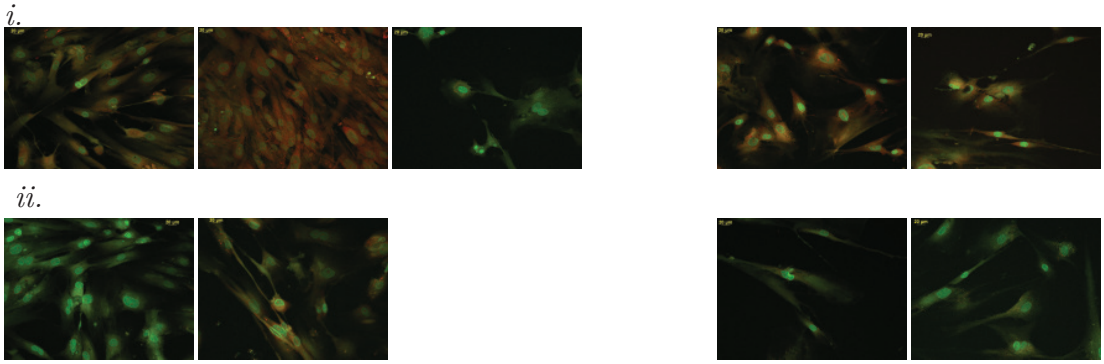


Figure 3.11.: Experiment 2 - controls.
i. Control proliferation under normal conditions at 2 days (left) and 5 days (right) *ii.* Control proliferation in medium containing NaSa at $1x10^{-6}$ M at 2 days (left) and 5 days (right)

When looking at the data for NaSa-C then there is a small peak in enzyme activity at 2 days and a small dip at 5 days when compared to other samples. Although these differences are small, if they are representative they could indicate that dying cells were removed with the change in medium at 2 days and the subsequent reduced cell count would then lead to the lower result at 5 days. This would be concurrent with microscopy results as while some cells were observed to have seeded on NaSa-P, on NaSa-C there were very few viable cells and cell debris could be observed (Fig. 3.13). There was little or no adherence on the stainless steel contained within this sample which suggests that reduction in cell survival may be due to excessive NaSa levels in the growth medium, rather than the ppy surface itself. This experiment informed the design of the EC experiments which followed as toxic levels of NaSa in solution may obscure results.

Fig. 3.14 shows the crossover regions between polymer and metal for each of the SMC adherence assays (excluding the example already shown in Fig. 3.13 where no cells are present). This indicates that it is possible for cells to extend across the boundary between steel and ppy despite the change in surface.

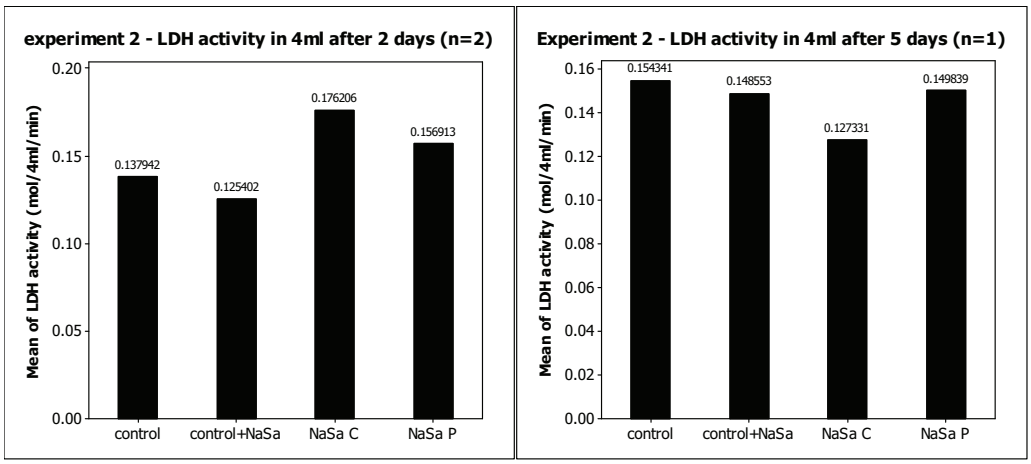


Figure 3.12.: Graphs showing levels of LDH activity in terms of NADH used, in medium taken from cell cultures used in Experiment 2 at 2 and 5 day time-points. Samples left to right:- *Control*: cells used experimentally grown under standard conditions. *Control + NaSa*: including NaSa solution at 1 x 10^6. *NaSa-P*. *NaSa-C*

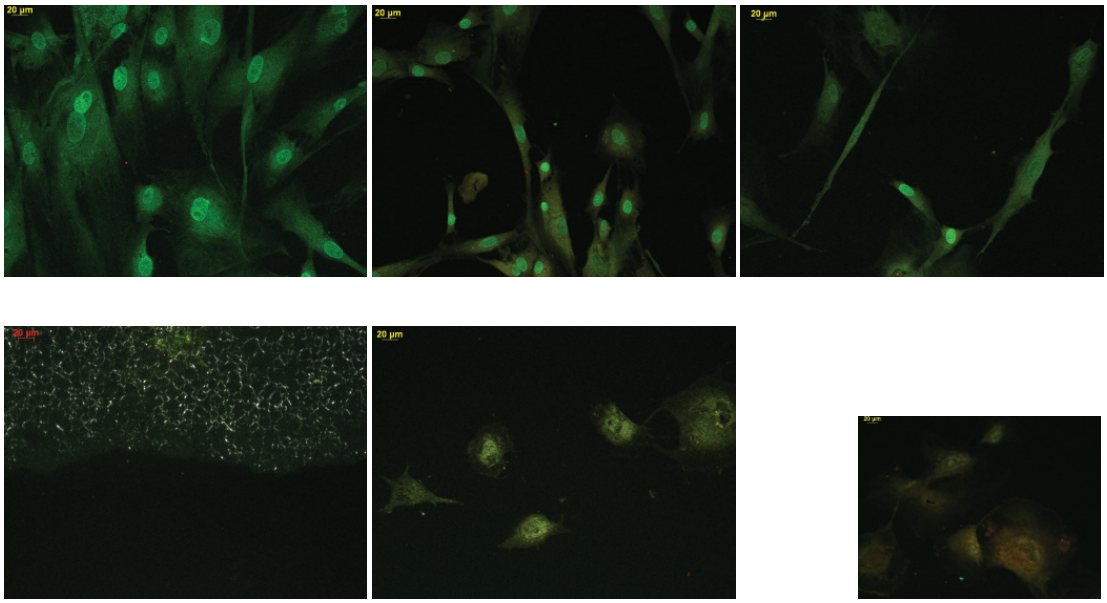
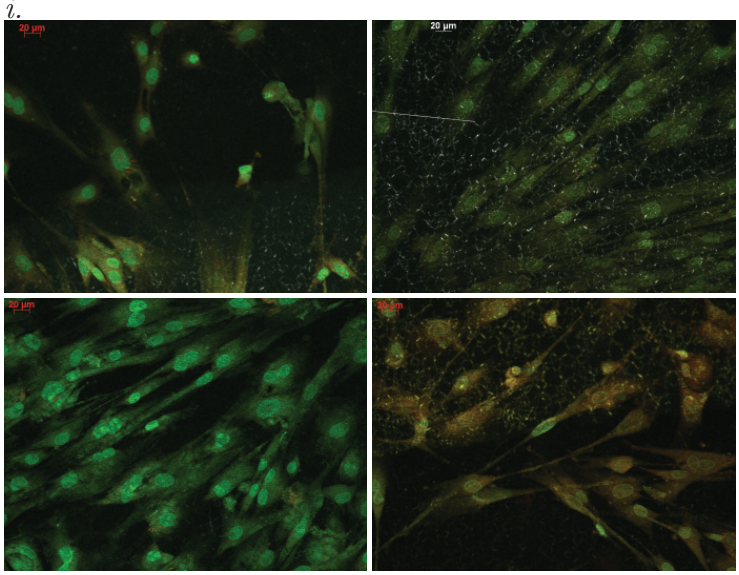
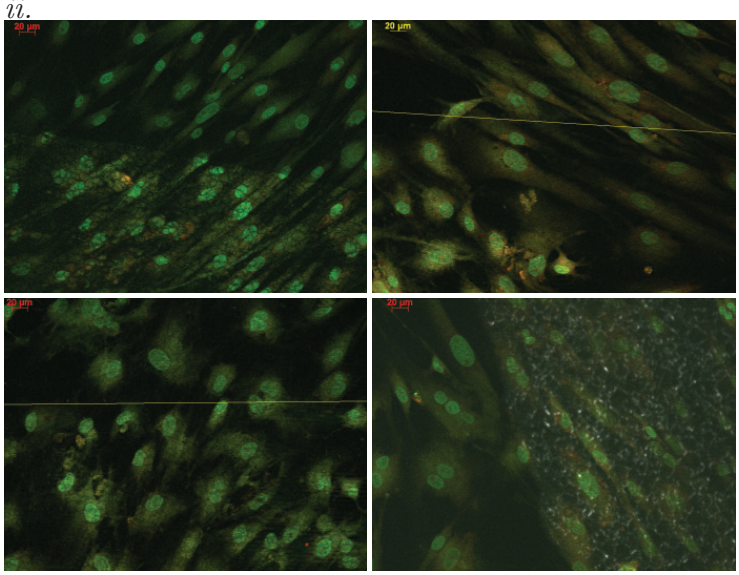


Figure 3.13.: adherence on ppy surfaces at 5 days, NaSa-P (top) and NaSa-C (bottom left). Also shown, poor cell survival on metal in the sample NaSa-C (bottom right)

i.



ii.



iii.

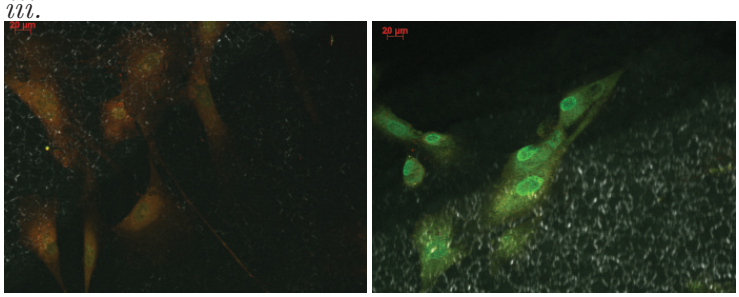


Figure 3.14.: Crossover regions between metal and polymer. DIC microscopy has been employed to reflect light from the surface. A clear line between materials is not always visible. Where it was visible by eye but not captured in the image, a line has been drawn using imaging software.

i. Experiment 1, 2 day:- *clockwise from top left:* NaSa-P, NaCl, NaSa-C, NaCl+NaSa

ii. Experiment 1, 5 day:- *as above*

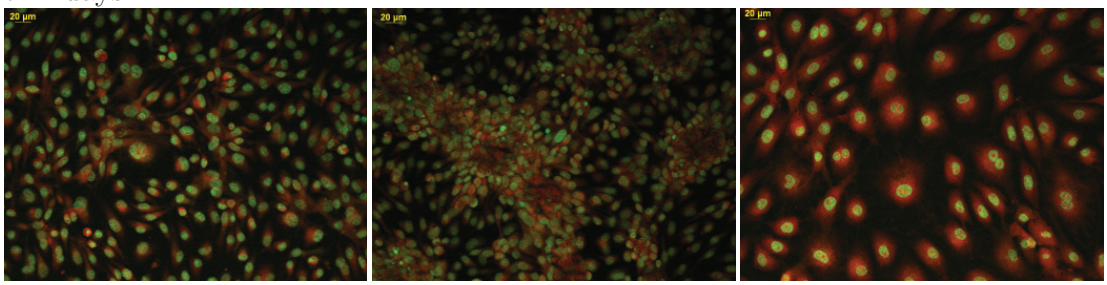
iii. Experiment 2 NaSa-P 2 day *(left)* and 5 day *(right)*

3.3. Endothelial cell proliferation and migration assay

To assess the suitability of ppy as a substrate for EC, these cells were also seeded on stainless steel plates part-coated with NaSa-P such as those shown in Fig. 3.1. They were then assessed in terms of cell adherence, viability and morphology as insec. 3.2, as well as their ability to migrate onto an area of polymer denuded using a cell scraper.

The progression of EC proliferation on tissue culture plastic, at two days and again at five days, is shown in Fig. 3.15. The monolayer is complete and the surface is close to confluence even after 2 days Even after 2 days, areas of low density were rare and in those areas cells are shown to be dividing. In some denser regions, cells appeared to form island-like structures within the monolayer. When cultured in medium containing $1x10^{-6}$M NaSa (Fig. 3.16), cell growth rates appeared to be slowed, with a reduced cell density being observed. After 5 days a greater range of morphological variation could be seen, with some clumping. In some cultures, morphology suggested that a small number of SMC may also be present although this cannot be confirmed. In all cultures majority of cells were observed within the expected cobblestone morphology.

i. 2 days



ii. 5 days

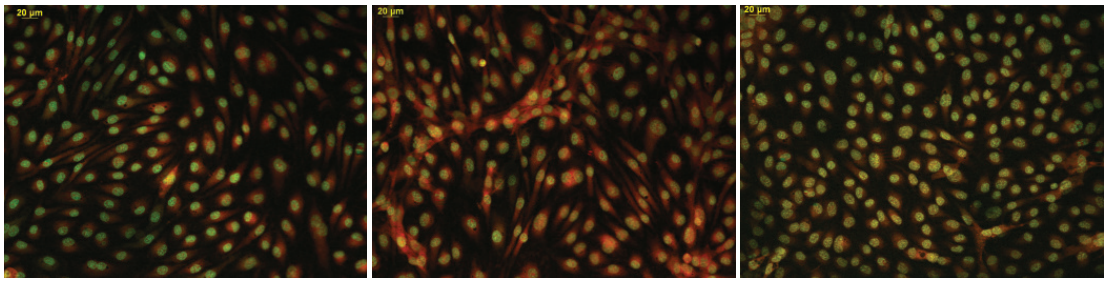
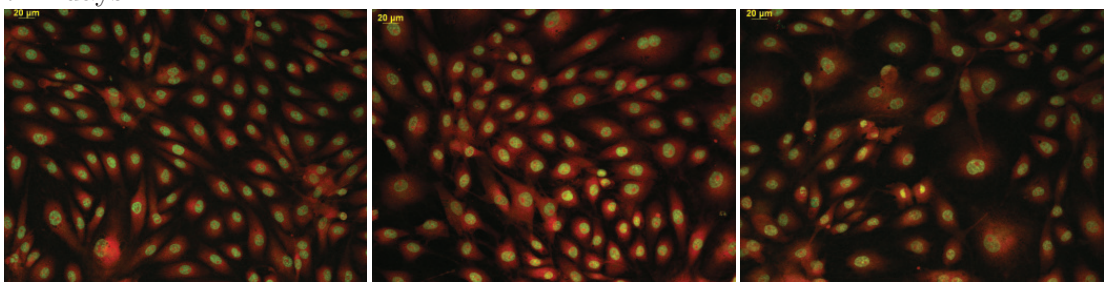


Figure 3.15.: Control proliferation of EC under normal conditions after 2 and 5 days.

i. 2 days:- left to right: Normal proliferation. Island-like structures. Area of lower density (rare)

ii. 5 days:- left to right: Normal proliferation. SMC-like morphology. Slightly denser, cobblestone morphology

i. 2 days



ii. 5 days

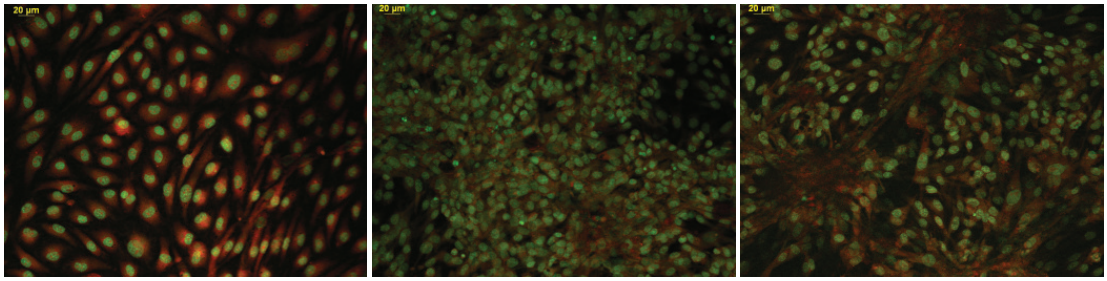
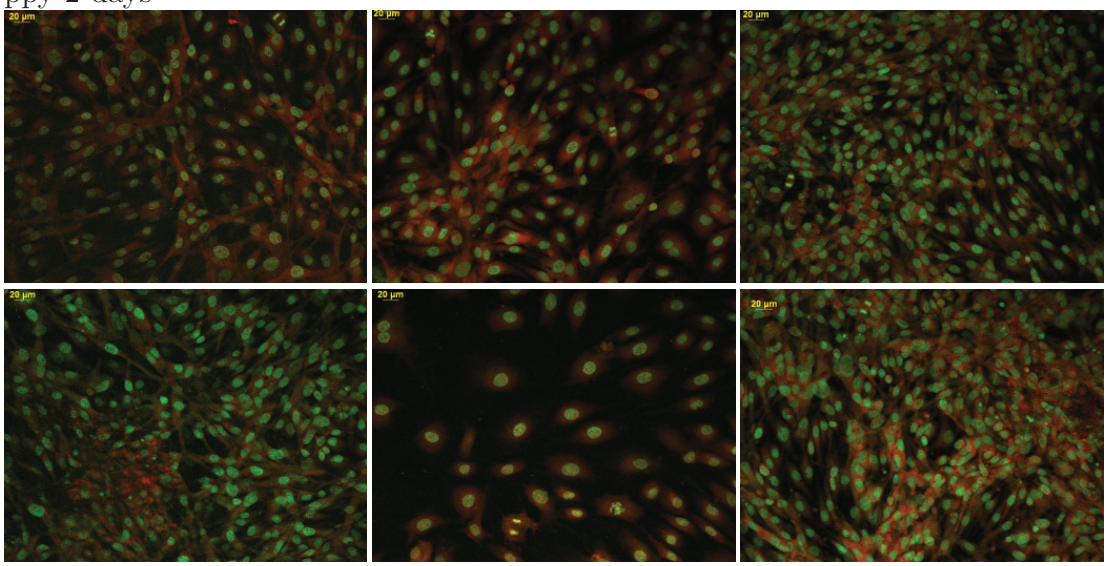


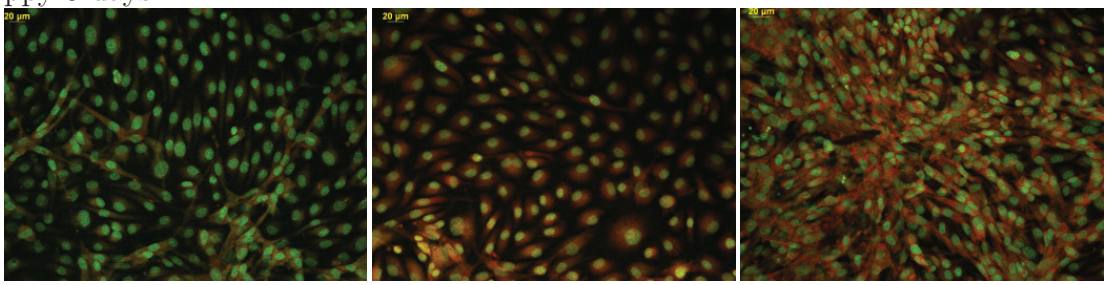
Figure 3.16.: Control proliferation of EC in medium containing 1x10^{-6}M NaSa after 2 and 5 days.
i. 2 days:- left to right: Normal proliferation. Less dense, cobblestone. Area of lower density
ii. 5 days:- left to right: Cobblestone, some elongation. Very dense. Dense, some clumping

EC showed an encouraging capacity for proliferation on the NaSa-P surface, with the full surface area of the polymer covered with a monolayer after 2 days. It cannot be ruled out that the confluence observed may be in part due to seeding density. However, population growth does seem to have been more successful on the ppy than was seen in the control sample containing NaSa and further to this, dividing cells are apparent on the polymer, particularly in areas of lower density. Some variation in density and morphology was seen and is represented in Fig. 3.17. This includes clumping similar to initial experiments involving SMC and, as with the control samples, some cells displaying a morphology which may indicate SMC presence. However, most regions of the polymer showed cells adopting the classic cobblestone morphology.

ppy 2 days



ppy 5 days



metal 2 days (left) and 5 days (right)

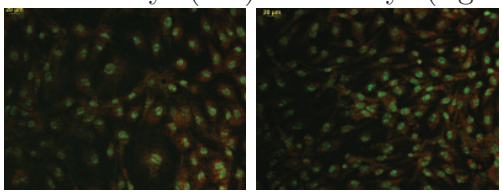


Figure 3.17.: adherence of EC on ppy and steel surfaces at 2 and 5 day time points. All ppy NaSa-P.
Density and morphology shows some variation as illustrated, but with no part of the polymer (excluding the manually scraped area) left free of cells. In all cases the cobblestone morphology was the most prevalent. Note that actively dividing cells are visible at both 2 and 5 days in the less densely populated regions of the polymer surface.

LDH assays show all cultures to be viable, with very low levels of enzyme activity detected. Positive controls and negative control samples were produced to establish maximum and minimum levels of LDH activity. These values were much lower than those seen in SMC culture. Fig. 3.18 shows these control values together with sample values. These were obtained as in Fig. 3.9 however in this case they have been included in the same graph to illustrate that compared with a positive control, all sample values were close to zero.

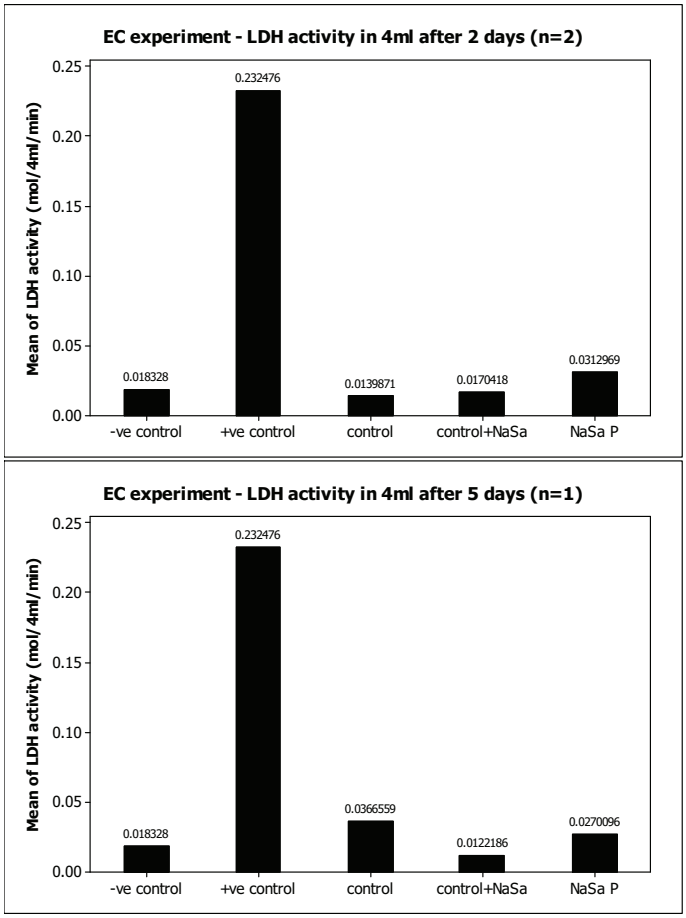


Figure 3.18.: Graphs showing levels of LDH activity in terms of NADH used, in medium taken from cell cultures used in EC experiments at 2 and 5 day timepoints. *Negative control*: duplicate samples grown over 3 days, tested for LDH release, then killed to establish a maximum LDH level (*positive control*). Samples left to right:- *Control*: cells used experimentally grown under standard conditions. *Control + NaSa*: including NaSa solution at 1 x 10^{-6}. *NaSa-P*.

No change in cell adherence was observed at the boundary between metal and polymer on these samples. This is shown in Fig. 3.19.

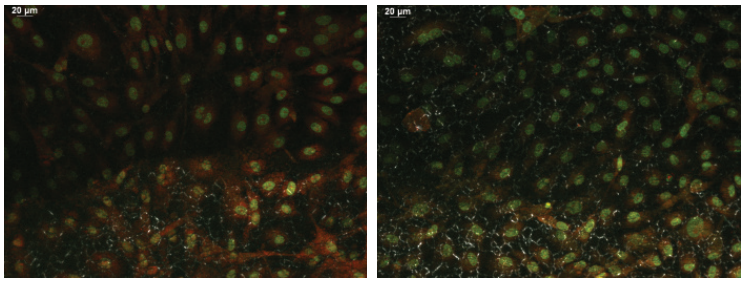
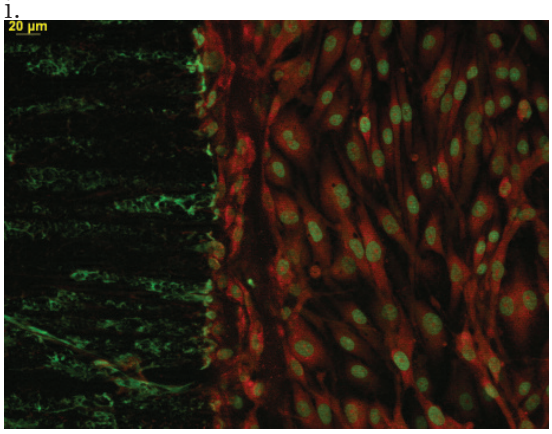


Figure 3.19.: EC growth over crossover regions between metal and polymer. 2 day *(left)* and 5 day *(right)*

Results of the migration assay are show in Fig. 3.20. When samples were fixed immediately after scraping a clear line can be seen with a denuded area to the left and cell monolayer to the right. However when samples subjected to the same scraping treatment were reincubated for a further 3 days in fresh medium there is no longer a clear break between the two regions and cells appear to be moving out onto the denuded area. Unfortunately in this assay it was not possible to identify the point at which the break would have been. This is in theory could indicate that cells are able to redistribute on the surface, although practically it means that migration can not be measured in terms of distance. Interestingly, cells also appear to have migrated from the bottom edge of the plate. This is likely to be from an area missed by the cell scraper rather than from the environment surrounding although again no clear divide is visible and cells become more elongated and increasingly spaced out towards the centre of the plate.

i.



ii.

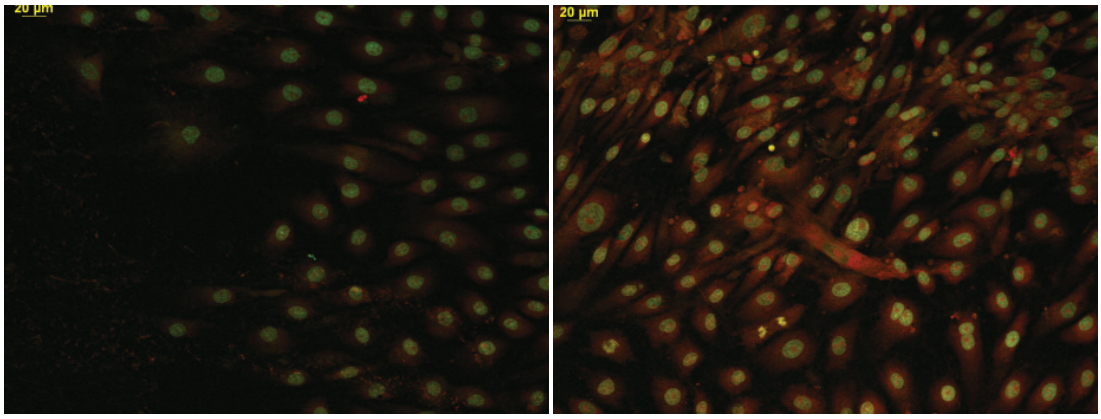


Figure 3.20.: Migration Assay

i. Image showing the clear line made by the cell scraper at day 2 with the denuded area to the left.

ii. Images showing the edge between the denuded area and the monolayer *(left)* and apparent migration from the bottom (microscope image inverted) of the polymer sample *(right)* at day 5.

3.4. Scanning Electron Microscopy(SEM)

To examine microtopographical features of the ppy and stainless steel surfaces, both trial and experimental samples were examined using SEM. Interestingly, despite differences in appearance visually, surfaces were consistently smooth regardless of synthesis conditions, and followed grain boundaries on the stainless steel. Images of NaSa-P and NaSa-C are shown in Fig. 3.21, alongside examples with variation in levels of cellular adherence but consistent topography. Areas of poor cell growth also showed this topography (not shown - see sec. A.3). Some rough areas were identified on a trial sample of NaCl-P ppy and it was thought this might contribute to the high levels of cell proliferation seen in some regions of NaCl-P surfaces, however when experimental samples were examined this proved not to be the case(Fig. 3.22). The ability to image cells on polymer and steel surfaces was unexpected as cellular samples can be hard to maintain within a vacuum. The fact that the cells can be observed gives an extra dimension to the images, illustrating the relationship between these SEM images and the characteristics observed by fluorescence microscopy.

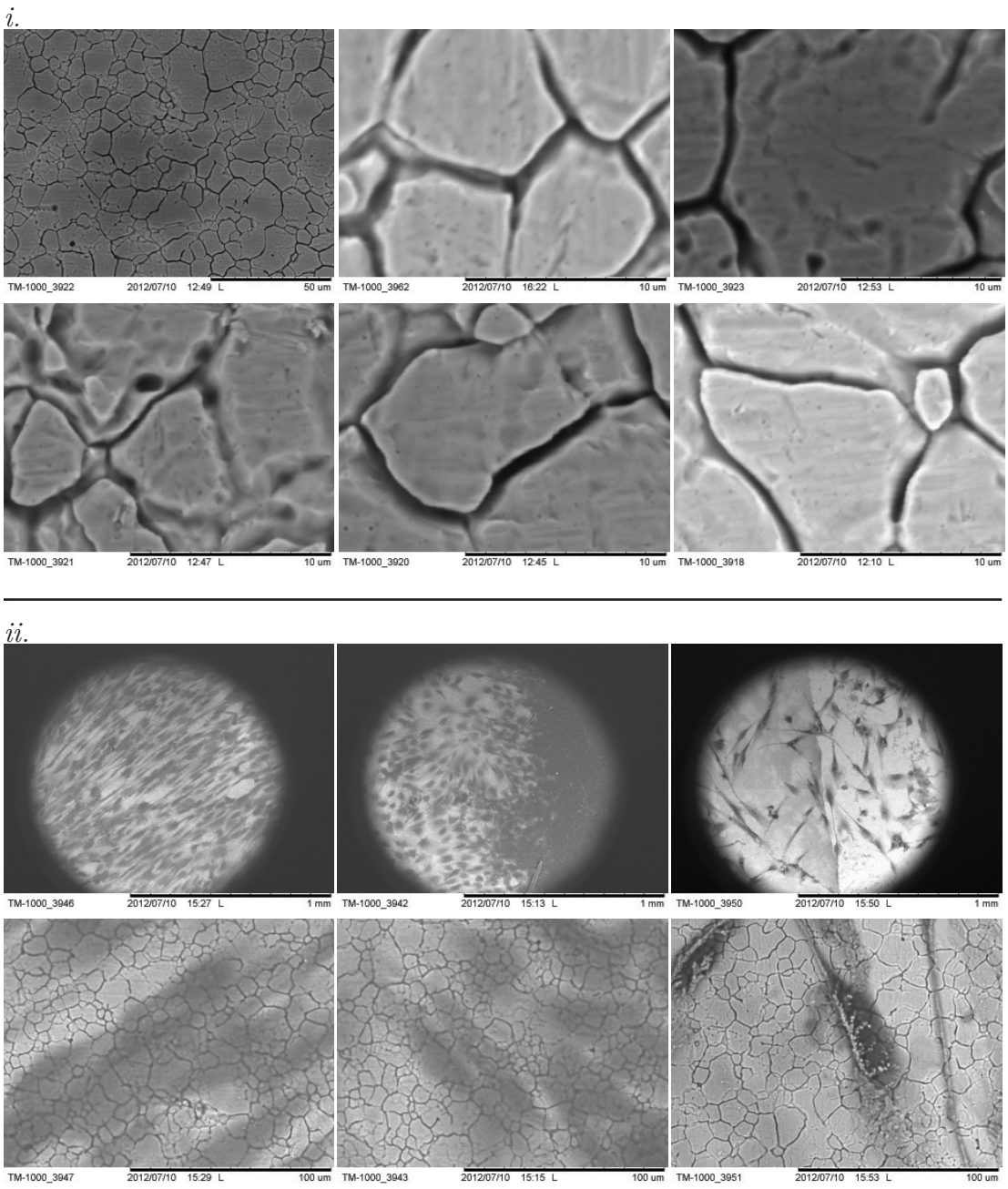


Figure 3.21.: SEM Images of NaSa-P and NaSa-C.

i. top row left to right: *NaSa-P x1200 showing unevenness in surface reflectivity, NaSa-P x1000 (lighter region), NaSa-P x8000(darker region),*

i. bottom row left to right (all at x10000): *NaSa-P, crossover point (ppy left/steel right), stainless streel*

ii. left: *healthy growth on NaSa-C at x100 and x1000*

ii. centre: *clumping on NaSa-C at x100 and x1000*

ii. right: *crossover region (NaSa-C left, steel right) at x100 and x1000*

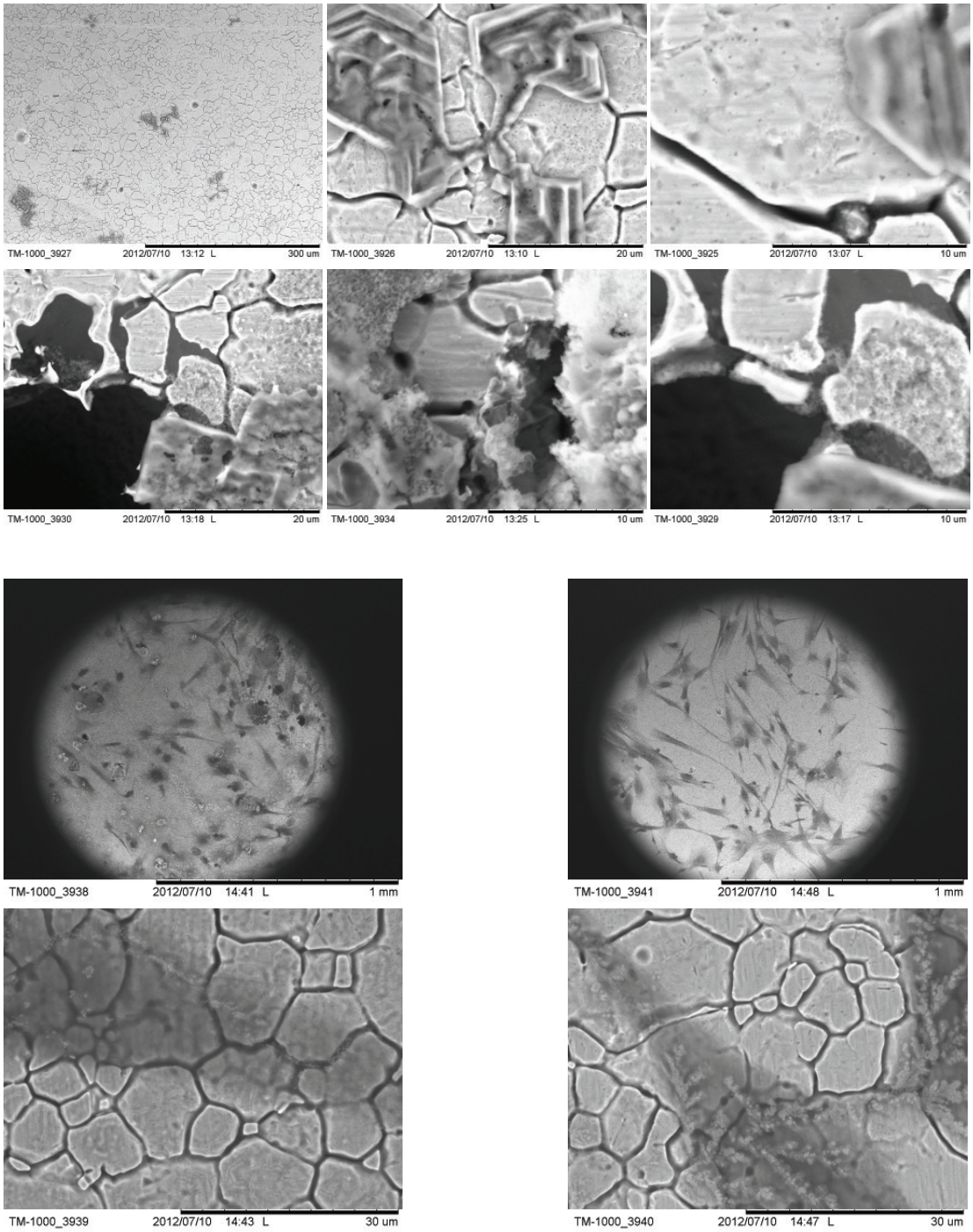


Figure 3.22.: SEM images of NaCl-P.
Top: images showing surface variation in NaCl-P trial sample, magnification increasing left to right.
Bottom left: images at 100x and 3000x of cells growing on polymer surface
Bottom right: images at 100x and 3000x of cells growing on metal surface

4. Discussion

4.1. Overview

This study aimed to monitor the growth of vascular cells on a Ppy surface, with variation in topography achieved through voltage modulation during electropolymerisation. Ppy surfaces synthesised by using sodium salicylate as a dopant ion and eluted drug was presented to SMC and EC in solution and their responses gauged in terms of confluence, morphology and viability

Ppy films were synthesised successfully on stainless steel plates using salicylate as a dopant ion, although an observable variation in surface topography was not achieved. The drug elution capability of coatings on stainless steel wires have been demonstrated by Anna Galluzzo in a thesis project run in parallel to this study and so it is clear that these ppy surfaces present eluted salicylate drug to cells adherent to the polymer surface. Both EC and SMC were observed to grow to a high density in their respective characteristic morphologies on ppy, with low LDH levels indicating a high level of viability. In these respects the experimental aims set within this project have been met.

4.2. Experimental hypotheses

4.2.1. The translation of synthesis parameters from steel wires to foils requires optimisation

Synthesis conditions were based on protocols established prior to the start of this work to optimise polymer deposition while avoiding overoxidation of the polymer. These protocols were designed for use in the coating of stainless steel wires and were retained in consideration of time constraints and and maintaining consistency with parallel projects. The first question asked of this study was therefore to what extent were the settings used transferable for use on steel foils.

In practice, these protocols for wire coatings did not transfer well to the asymmetric 3D structure and in the case of NaCl as a dopant ion, it was difficult to acheive a coating consistent enough to use experimentally, with poor deposition and some oxidation of the working electrode observed. Going forward in these experiments it will be important to optimise the parameters used to synthesise ppy films reliably when the dimensions of the working electrode are varied.

4.2.2. Viable vascular cell cultures were achieved on a polypyrrole surface

Both SMC and EC were observed to grow on polypyrrole surfaces. While SMC showed a high degree of variability and was not consistently confluent, EC formed a fully confluent monolayer, albeit with some morphological variation observed. The viability of cells in culture is confirmed by the LDH assays which demonstrate high levels of cell survival, particularly in the EC population.

Full serum is used here as a pre-treatment but it does not contain anything that is not already in the growth medium and levels of given proteins have not been altered. This is a technique which has been used by others and has been shown to be effective in promoting adhesion[15]. Protein adsorption is a near-instantateous process, with an approximately exponential decrease in the rate of deposition, dependent on factors relating to the polymer and the proteins present[72, 6]. This means that a layer of protein will have adsorbed and begun to equilibrate following an incubation period of 1-2 hours. The composition of the protein layer will change initially as molecules compete to adsorb to the surface, influenced by properties such as size and charge as well as binding affinity and other molecular interactions. It has been shown mathematically that these proteins can remain in a state of flux for up to 10^6 years[22]. The adsorption and equilibration rates of serum proteins depend on the character of the polymer and so it may be possible to optimise the incubation time to maximise equilibration once further characteristics of the polymer are known. However, as this step was included to allow proteins time to become established before cells were introduced then optimisation may not be necessary.

Prior studies of endothelial cells grown on ppy have been successful however the surfaces have been functionalised in some way, either by hyaluronic acid, heparin or carboxylic acid incorporation or conjugation, or with artificially raised levels of a given plasma protein, such as fibronectin or vitronectin[32, 48, 31, 5]. Additionally few of these have been vascular endothelial cells with many groups

focusing on human umbilical vein endothelial cells (HUVEC). If these assays were repeated to form a data set of statistical merit, and if these were successful, then it would demonstrate that EC can grow on ppy not only without specific functionalisation but in the presence of a drug known to reduce proliferative capacity.

4.2.3. Topographical influence on cell type

Here we hypothesised that proliferation of SMC and EC would be influenced by variation in surface topography achieved through use of different synthesis techniques. Despite successful synthesis of ppy films under different conditions and, to a lesser extent, dopant ions this was something this work was unable to address due to the topographical uniformity seen between under the SEM, shown and discussed in sec. 3.4. The question of topographical impact remains an important one and possible approaches to be considered will be discussed in sec. 4.3

The uniformity of the surfaces generated was surprising as examples in the literature show a wide range of surfaces which can be generated by modulation of synthesis technique and conditions. NaSa doped polypyrrole has been reported as having a characteristic 'cauliflower' morphology[2] and this is what was expected. However, it is known that thickness affects the topography of film deposition and it is possible that the films achieved here were simply too thin to have an estabilished morphology[5].

What was particularly striking about the SEM images of the polymer, other than uniformity, was that their pattern can be seen to mimic the grain boundaries of the stainless steel. Grain boundaries form where crystals within the metal alloys meet. Within a crystal atoms will hold a specific orientation, which may differ from adjacent crystals. The boundary is where these two orientations meet. Consequently these lines are points of disorder as the atoms are not in the same orientation, and so are more loosely packed and hold a higher internal energy[27]. These grain lines are also visible in images using DIC microscopy which identifies 3D physical features so it may be that if the polymer is very thin that it has not yet filled the slight indents between the crystals. However as these areas of increased energy will display different electronic properties it is possible that this may also influence deposition patterns.

Though it would have been useful to assess the effect of topography on cell growth, this result allows for the assessment of ppy surfaces in the absence of gross to-

pograpical differences when compared with the stainless steel control surface. Removal of this variable simplifies the experimental system thus making it possible to achieve a fair, direct comparison of cell growth on steel and polymer.

4.2.4. EC migration

As EC migration is thought to be of major importance in healing following stenting, a further key hypothesis of this study was that ppy would be a substrate on which EC would migrate. As discussed in sec. 3.3, while there were indications that migration was occurring, these were not quantifiable due to a lack of definition of the point from which the monolayer had been denuded. However, this itself suggests that the EC were capable of redistribution on the polymer surface. Furthermore, dividing cells could be identified showing that cells were able to thrive and proliferate. This experiment was n=1 so the signifance of this result is limited, however it may provide a positive indication for further research.

4.2.5. Influence of salicylate incorporation into polymers on cell proliferation

As stated in sec. 4.2.2, EC formed a confluent monolayer on the NaSa-P surface on which they were seeded. The density of this monolayer appeared higher than was seen in the control sample containing salicylate and to have a comparable level of morphological variation. SMC cultures were observed to grown on NaSa-P, though with a greater level of inconsistency than was seen in the EC population.

High doses of salicylate can halt SMC proliferation by arresting the cell cycle at the G_1-S phase. The dose required for this effect to occur is reported to be 0.5M which is much higher than the $1x10^{-6}$M dose administered to cells in control cultures[52]. However, in these experiments growth did seem to be affected by NaSa presence even at these low levels. These results can be seen for SMC in Fig. 3.3 and Fig. 3.4. These effects included clumping and regions of poor growth, neither of which were observed in control samples without NaSa. For EC, shown in Fig. 3.15 and Fig. 3.16, the influence seemed to be in proliferation rate primarily, though again there were some areas of clumping after 5 days. The potential shown by NaSa as a therapeutic agent against vascular SMC hyperproliferation has been attributed to effects on the proline-rich tyrosine kinase 2 (PYK2) pathway[63, 94]. Part of the role of this pathway is in reorganisation of the actin cytoskeleton and interplay between this and the pathways related to focal adhesion is critical to

regulation of growth, morphology and migration[17]. PYK2 has also been found to promote EC proliferation. One may therefore speculate that at these low levels some of the reduction in growth rate as well as morpological variation and clumping may be indicative of PYK2 interference.

Results of experiments using NaSa-P seemed to compare well against the control samples grown in an environment containing NaSa. However, results from Anna Galluzzo's MSc project on the drug elution profile of ppy surfaces suggests that NaSa-C elutes much higher levels of drug when compared with NaSa-P[28]. Results from experiment 2 showed poor survival both on the NaSa-C polymer and steel. Negative effects on the cells observedthe NaSa-C ppy could be due to localised high concentrations at the cell:polymer interface, however as cells were also shown to be inibited on the steel, this indicates that levels in the growth medium were high enough to become toxic to cells.

The inhibition of cell proliferation on the NaSa-C surface in experiment 2 was more severe than the NaSa-C surface used in experiment 1, which was produced following the same procedure. However, the initial surfaces generated and used for experiment 1 used pyrrole monomer stock solution which had been opened some months previously and while it had been stored under argon to prevent oxidation, for synthesis of these surfaces a slight discolouration was noted. As darkening of the solution is likely to represent part-polymerisation of monomers prior to experimental use, a new stock solution was acquired which did not show discolouration and this was used for experiment 2 and the subsequent EC experiments. While the visual appearance of the films generated did not change between the two solutions and there was no noticeable difference during polymer synthesis, results from Anna Galluzzo's project on the drug elution profile of these surfaces show that high concentrations of salicylate were eluted from NaSa-C when the new pyrrole stock solution was used. This perhaps reflects a higher proportion of monomers and dimers in solution, taking up a larger number of salicylate ions as their reaction progresses and provides a valid explanation for the discrepancy between results on NaSa-C in experiments 1 and 2.

4.3. Future work

4.3.1. Optimisation of electropolymerisation for stainless steel plates

A key limitation of this project undeniably was the application of protocols optimised for stainless steel wires to stainless steel plates.

These protocols, based on the work of Arbizzani[4], focused on voltage modulation whereas a common approach is to calculate the current density at the point where the polymerisation occurs, in terms of area[81, 41, 2]. There are also suggestions in the literature that the optimal molarities for NaSa and pyrrole monomer would be higher than used here; Hermelin et al give 2M and 0.5M for the solutions respectively, at pH 5 at room temperature[41]. It may also be beneficial for the anode to be rotated within the electrochemical cell to ensure it is coated evenly and so is more predictable.

By looking at current density at the anode and by varying monomer and dopant concentration, as well as thickness generated over time it should be possible to optimise film production for this environment in a way which can be scaled up or down to accommodate application in device prototyping. It would then be worthwhile to take these more reliably generated surfaces and see how they perform in the same vascular cell assays used in this project.

4.3.2. Achievement of a NaSa-free ppy control

As discussed above, protocol optimisation would enable the achievement of a more consistent NaSa-free ppy control than was possible here. However, it would also be interesting to deplete samples of the more successful NaSa-eluting polypyrole of the drug they contain, in order to establish the effects of the polymer independently of NaSa pharmacology while retaining consistency.

4.3.3. Full surface characterisation

SEM, while achieving a high resolution when compared with standard visualisation methods, is still relatively crude in the sense that it can only measure surface variation to a level of microtopographical variation. For these surfaces to be fully compared it would be necessary to scan them using atomic force microscopy (AFM). This would enable comparisons to be drawn between surfaces

at a nanotopographical level. Variation has previously been observed at this level using AFM on ppy films synthesised using different dopant ions[33, 32]. It would be important to understand the extent to which this variation affects cell growth as nanoscale variation can influence the plasma proteins which are more likely to be adsorbed to a given surface, which in turn is implicated in the adhesion of given cell types[6, 32].

In addition to the physical characterisation of the ppy surface it would be very important to look at the chemical profile it presents, as well as the composition and orientation of of the adsorbed proteins which would go to form the cell-facing environment. Increased binding of proteins such as vitronectin would be beneficial to endothelial cells, for example[6, 32]. Equally, a more hydrophobic surface may discourage non-specific binding but expose an increased number of molecular binding sites incorporated into the polymer designed to attract and retain cells, for example. This is discussed by Garner et al in the context of heparin-conjugated polypyrrole[32].

4.3.4. Characterisation of variation in morphology

In sec. 4.2.5 it is suggested that clumping may be due to NaSa intereference in the PYK2 pathway. One way to easily assess if morphological differences are related to actin dysfunction would be to carry out DAPI/FITC-phalloidin staining. This fluorescence microscopy technique allows visualisation of the actin cytoskeleton and so may be a valuable assay to this project in the future.

It was also suggested in sec. 3.3 that some of the more elongated cells may represent SMC within the cell population. Therefore it would be important to use marker proteins such as SMC alpha-actin to determine cell phenotype.

4.3.5. Quantifiable EC migration assay

In sec. 4.2.4, the limitations of the existing migration assay were analysed alongside the benefits of a quantifiable migration assay. It would be reasonably straightforward to mark a point on the base of the tissue plate from which the polymer was denuded. That way it would be possible to measure migration distance and cell density beyond that point. It is also important to note that this assay was carried out at a cell seeding density which was inappropriately high for the level of proliferation which was subsequently observed. This is concurrent with

experiences of the cells in tissue culture where their return to confluence was consistently rapid over the short duration of their maintenance. However time constraints meant it was not possible to adjust or repeat the experiment following this observation.

4.3.6. Adhesion in a dynamic system

One limitation of this study is that it is static and so the shear stresses present in the in vivo environment are not represented. Focal adhesions, which mediate cell anchorage, can be influenced by shear forces[88]. Furthermore, adsorbed protein composition would be biased towards those which could retain the strongest bonds in a dynamic system[87]. As discussed in sec. 4.2.5 the influence of salicylate can also affect the balance between focal adhesion and PYK2 pathways in cytoskeletal regulation and so use of salicylate, unless it was to be considered as a drug for use in a prototype DES system, should be carefully considered.

4.3.7. Potential for EC preferential growth?

The target of much stent research activity in recent years has been the development of stents which inhibit smooth muscle cell proliferation whilst promoting recovery of the endothelial cell layer. These results tentatively suggest that on these thin NaSa-P coatings, EC could thrive whereas SMC were less successful. However, this is a hypothesis which would require a great deal of work to verify or disprove. An interesting next step following optimisation of surface coating and seeding densities would be to carry out a co-culture and quantify populations by specific staining or flow cytometric methods.

4.4. Application of these findings to a DES context

Many of the design criteria of any ppy-coated DES which could emerge from this preliminary study could potentially influence the successful seeding of vascular cells on a ppy coating. This is particularly true of the choice of dopant ion and drug incorporated. These results are demonstrated with salicylate which has been used previously in this context. It may be interesting to investigate the influence on vascular cell growth if a different drug is chosen.

One observation which may prove relevant to DES development is that in production of these thin coatings, the crossover area between steel and polymer did not act as a barrier to growing cells. This may be important as a boundary within the cell population of a newly healed vessel may introduce a site of vulnerability. It would be interesting to identify if this advantage continues to be observed when thicker films are used.

As the brittle nature of ppy would pose a potential risk in a DES context, it would also be important to research whether synthesis conditions and thickness affect coating durability.

4.5. Conclusion

The overall aim of this study was to assess the suitability of polypyrrole for use in the formulation of a novel, EC-supportive durable drug-polymer for stent applications. These experiments have shown ppy to be an effective growth substrate for vascular cells with the ability to support cell proliferation while acting as a drug delivery mechanism. This preliminary study demonstrates the considerable potential displayed by polypyrrole for use in a vascular environment and supports further research into the development of a polypyrrole-based drug-polymer for use in coronary stenting.

Acknowledgments

With sincere thanks to Dr Chris McCormick for offering me this fascinating and rewarding project, and for his time and effort throughout. Thanks also to Brian Cartlidge, Katie Henderson and all staff and students working in the level 4 laboratories of the Wolfson Building for their advice and support.

A. Additional images

A.1. Polymer coatings

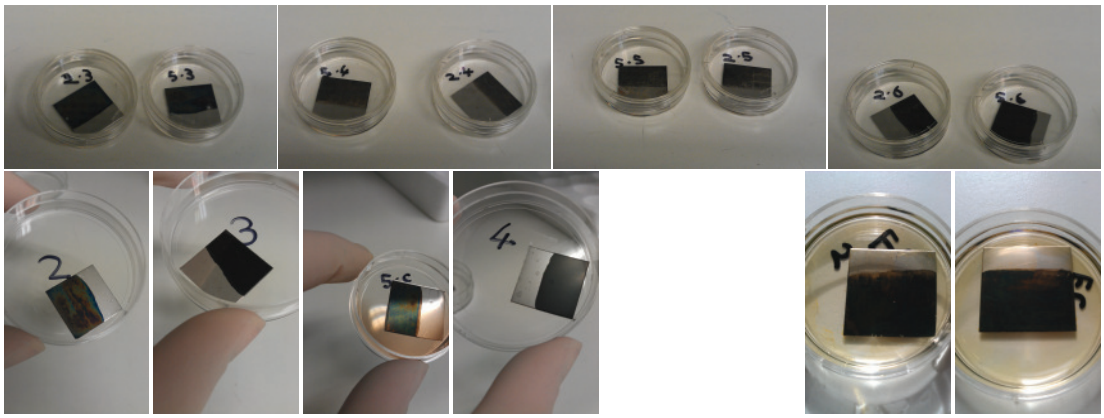


Figure A.1.: All polymer coatings used experimentally.
Top: Samples used in experiment 1. *2.3 and 5.3:* NaSa-P, *2.4, 5.4, 2.5, 5.5:* NaCl-P, *2.6, 5.6:* NaSa-C
Bottom left: Samples used in experiment 2. 2 day NaSa-P, 2 day NaSa-C, 5 day NaSa-P, 5 day NaSa-C
Bottom right: NaSa-P samples used in EC experiments *(2 day left, 5 day right)*

A.2. Fluorescence

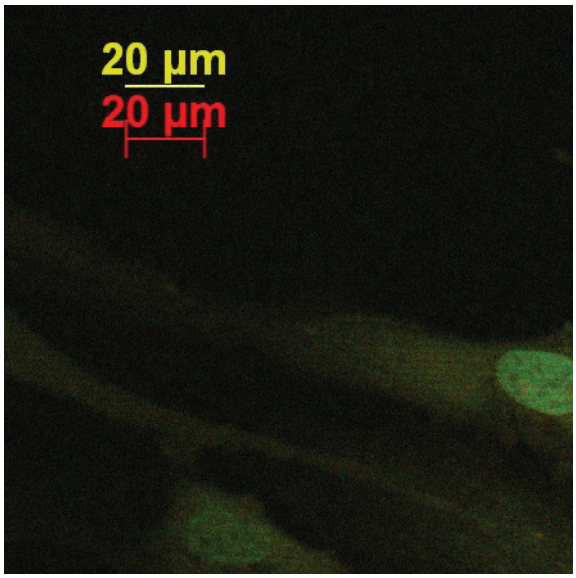
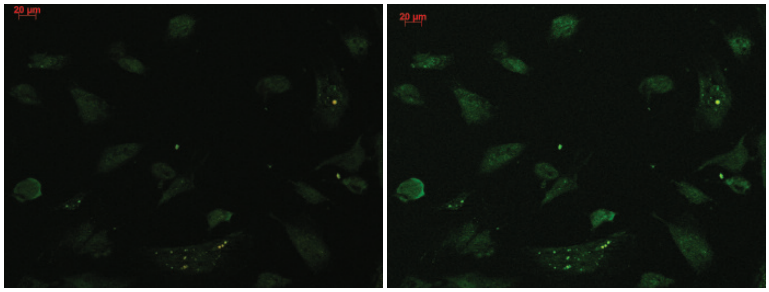


Figure A.2.: Some images required a scale bar to be added retrospectively. This was done using the Axiovision Lite software released by Carl Zeiss for home use. Distance was calibrated using pixel number, in with reference to equivalent scale bars which were already present (yellow) on images of the same size and magnification. The scale bar calibrated by pixel number is shown in red in images.

Figure A.3.: In Fig. 3.8 one image was adjusted using Microsoft image software to improve contrast. The change from the original image (left) is shown here.



A.3. Scanning Electron Microscopy

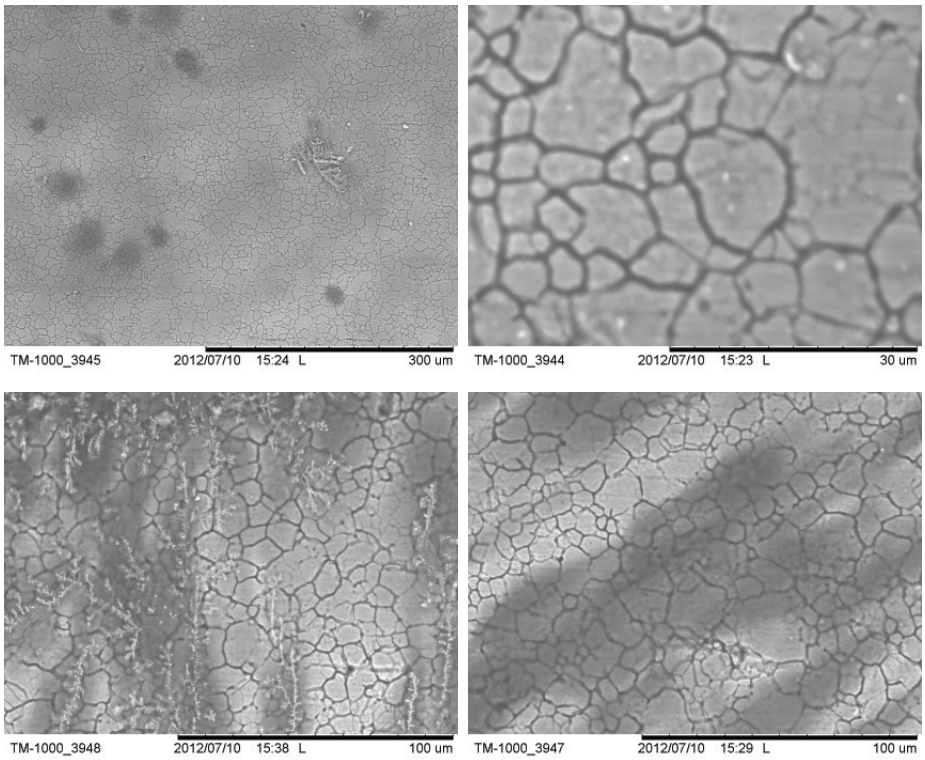


Figure A.4.: As discussed in sec. 3.4Areas of poor growth on NaSa-C (top) contrasted with areas of strong growth on the same polymer (bottom)

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