Product development specifications for a follicular sampling device for use in a human in-vitro fertilisation clinic

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Abstract:

The likelihood of pregnancy in human *in-vitro* fertilisation is heavily dependent on the condition of the embryos that are re-implanted into the patient's uterus. The condition of embryos is in turn dependent on the quality of the oocytes, from which they grew. It has been suggested previously that oocyte quality could be related to the level of dissolved oxygen in the ovarian follicle. The first objective of this work was to develop a set of product development specifications for a device that would be used routinely in a fertility clinic for sampling follicular fluid for dissolved oxygen determination. The second objective was to design and construct a prototype so that the relationship between dissolved oxygen and oocyte quality could be established.

A length of time was spent at two fertility clinics, one in Hamilton, New Zealand and one in Auckland, New Zealand. The experiences at these clinics, as well as technical constraints, were translated into a set of product development specifications. These specifications canvassed issues relating to cleanliness, potential damage to the oocyte and preservation of the dissolved gas equilibrium in the sample. A prototype device was designed and developed and found to be wanting in the clinical environment. Further clinical constraints were identified from this experience, allowing a second prototype device to be developed. This second device was found to be suitable for clinical use and it is anticipate that in the future the sampling device will re-emerge in a new, more suitable form, based on the specifications developed in this thesis.

Measurements of intra-follicular dissolved oxygen are on-going.

Acknowledgments

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I would like to thank the Beatles for the White Album.

The research for this thesis was approved after consideration by the Waikato Ethics Committee.

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Chapter 1. Introduction

Earth's increase, foison plenty,
Barns and garners never empty,
Vines with clustering bunches growing,
Plants with goodly burden bowing;

Spring come to you at the farthest
In the very end of harvest!
Scarcity and want shall shun you;
Ceres' blessing so is on you.

The Tempest IV, i. William Shakespeare

While Ferdinand and Miranda had the benefit of Ceres' blessing, it is often medical intervention, rather than divine intervention that is sought in the 21st century for issues of fertility. Since the birth of a child in 1978 (Steptoe & Edwards, 1978) as a result of *in vitro* fertilisation (IVF), an entire industry has grown to supply treatment to those affected by infertility.

This thesis describes the work carried out over a 12 month period directed towards the design of a product for use in the modern fertility clinic that would allow clinicians some insight into the conditions in which an egg had developed in a patient's ovary.

In vitro fertilisation is a treatment that is used to aid couples that are experiencing difficulties conceiving. The woman is usually given hormones that encourage her ovaries to produce more oocytes (eggs) than would occur during a normal ovarian cycle. These oocytes grow inside small (up to approximately 10ml) blisters, filled with fluid on the surface of the ovaries. These blisters are called follicles. At the appropriate time a clinician will extract the follicular fluid and oocytes and the oocytes will be cultured in a laboratory. The oocytes are fertilised *in vitro* and the resulting embryos are grown further. After a few days the embryos can be implanted into the woman's uterus, frozen or discarded.

During the culture process a large proportion of embryos will develop abnormalities or die. This is thought to be an effect of the oocytes from which the embryos were grown. The quality of the oocytes is thought to be influenced in part by the supply of blood and oxygen to the follicle. So, if we know the oxygen levels in the follicle, we may be able to infer information regarding the quality of the oocyte. The effect of implanting low

grade embryos is a reduced likelihood of pregnancy and live birth. Thus, if the clinician has information regarding the oocytes, then the patient's treatment can be managed to improve the chances of a successful treatment.

1.1 Objectives

The primary objective of this project is to develop a set of product specifications that will help to guide the design of a device for sampling follicular fluid. The samples will be used to determine the dissolved oxygen content of the follicular fluid.

The secondary objective of this project is to develop a prototype device and use it in a fertility clinic. This will help to establish a correlation between dissolved intra-follicular oxygen and oocyte developmental competence, and will help to validate and refine the specifications from the primary objective.

1.2 Product specifications

Product specifications are a set of constraints that describe what a product is going to achieve. It is not a description of what the product *is* or how it works, it is a description of what it *does*. A specification is a measurable parameter that meets a need that has been expressed by a customer or identified through research.

The sampling system is to be composed of several modules (for technical reasons that are disused later in section 2.6.3). The first is the module that will interface with the surgical equipment that is used to extract the oocytes and will divert part of follicular fluid away from the bulk flow as a sample. The second part is a container that will hold the sample after it has been removed from the bulk of the follicular fluid. The third part of the system is the instrument that will be used to determine the dissolved oxygen levels in the fluid and the final part is the set of operating procedures that determines how the device will be prepared to clinical standards, used and treated after use. A detailed understanding of the physiology surrounding IVF treatment is needed in order to describe an appropriate system.

Chapter 2. Review of the literature

> And so she said to Abram, "The Lord has kept me from having children. Why don't you sleep with my slave-girl? Perhaps she can have a child for me." Abram agreed with what Sarai said.
>
> Genesis 16:2

It wasn't until the ripe age of 90 that Sarai bore a child (Isaac) to Abram herself, as the result of a divine act. Clearly recognition of infertility, and some of the anxieties associated with this condition, can be found in some of the earliest writings. This literature review deals with more contemporary publications.

In order to achieve the objectives stated in section 1.1, there are several fields of learning that need to be reviewed. These topics are the nature and physiology of ovaries, follicular growth and oocyte maturation (section 2.1), the IVF process (section 2.2), an overview of previous efforts to predict developmental capacity of human oocytes based on follicular properties (section 2.3),the product development process and how it can be applied to biomedical devices (section 2.4) and finally, a description of the field of dissolved oxygen determination in human bodily fluids (sections 2.5 and 2.6).

2.1 Ovarian physiology and the follicular cycle

To give this work some background, an overview of the nature of the ovaries, their function and their behaviour must be considered. Even though their presence was likely to have been noticed by Hippocrates (460-370BC) during his extensive examination of the uterus, the ovaries were largely overlooked as being the source of the elements required to form a foetus for several centuries. Galen (130-200AD) proposed that the ovaries contributed some sort of blood filtrate, via the fallopian tubes, to the uterus, which would mix with male semen and coagulate, forming a foetus. Regnier de Graaf (1641-1673) regarded the vesicular structures of the ovaries (ie. the tertiary follicles) to be the eggs (analogous to bird eggs) which, after being fertilised, would undergo embryonic and foetal development. Although he was not the discoverer of mammalian eggs (that honour belongs to William Cruikshank), von Baer found the source of mammalian eggs (oocytes) in 1827. More detailed accounts of ovarian history are in ready supply, such those of Short (1977), O'Dowd & Philipp (1994) and of Hunter (2003).

2.1.1 Follicular structure and growth

Each germ cell (oocyte) is contained inside a structure called a follicle in the ovarian cortex (in the outer layer of the ovary). At birth each ovary contains about 500,000 follicles, about 83000 at puberty, 30,000 at age 35 and <1000 at age 50. 400-500 are used in ovulation. Most of them degenerate by a process called atresia before the follicle has fully matured. The peak in germ cell numbers occurs in the prenatal stage, approximately 6 months after conception. The significance of this is that each female has a number of oocytes at birth and, unlike spermatozoa in males, no more will be produced.

Most of the follicles in an ovary are primordial follicles. They are about 50μm in diameter, lie in the periphery of the cortex and consist of an oocyte and a single layer of granulosa cells, known as the membrana granulosa. Granulosa cells are gland cells, which means that they are capable of producing hormones and secreting these hormones into the surrounding space or into blood stream for distribution around the body. At this stage the granulosa cells are pressed flat against the oocyte.

2.1.2 Structure of the human ovaries

The ovaries are the gonads in the female, that is, a gland that produces germ cells, or gametes. The ovaries are found in the human female lying on each side of the upper pelvic cavity (figure 2.1), against the back of the pelvic wall, near the uterus. Each ovary "resembles an almond in size and shape" (Anderson, 2002). The ovary is covered in a thin layer called the ovarian epithelium, below which is dense tissue called the ovarian cortex and less dense tissue, called the ovarian medulla. The medulla contains blood vessels, lymphatic vessels and nerves. The medulla and cortex form the ovarian stroma (connective tissue framework). The female germ cells are in the cortex. The ovaries are connected to the uterus by the mesovarium and broad ligament, and are in contact with the fallopian tubes via the infundibula, formed by the fimbriae tubae at the distal ends of the fallopian salpinges. The ovaries are heavily vascularised and are supplied with blood from the ovarian artery.

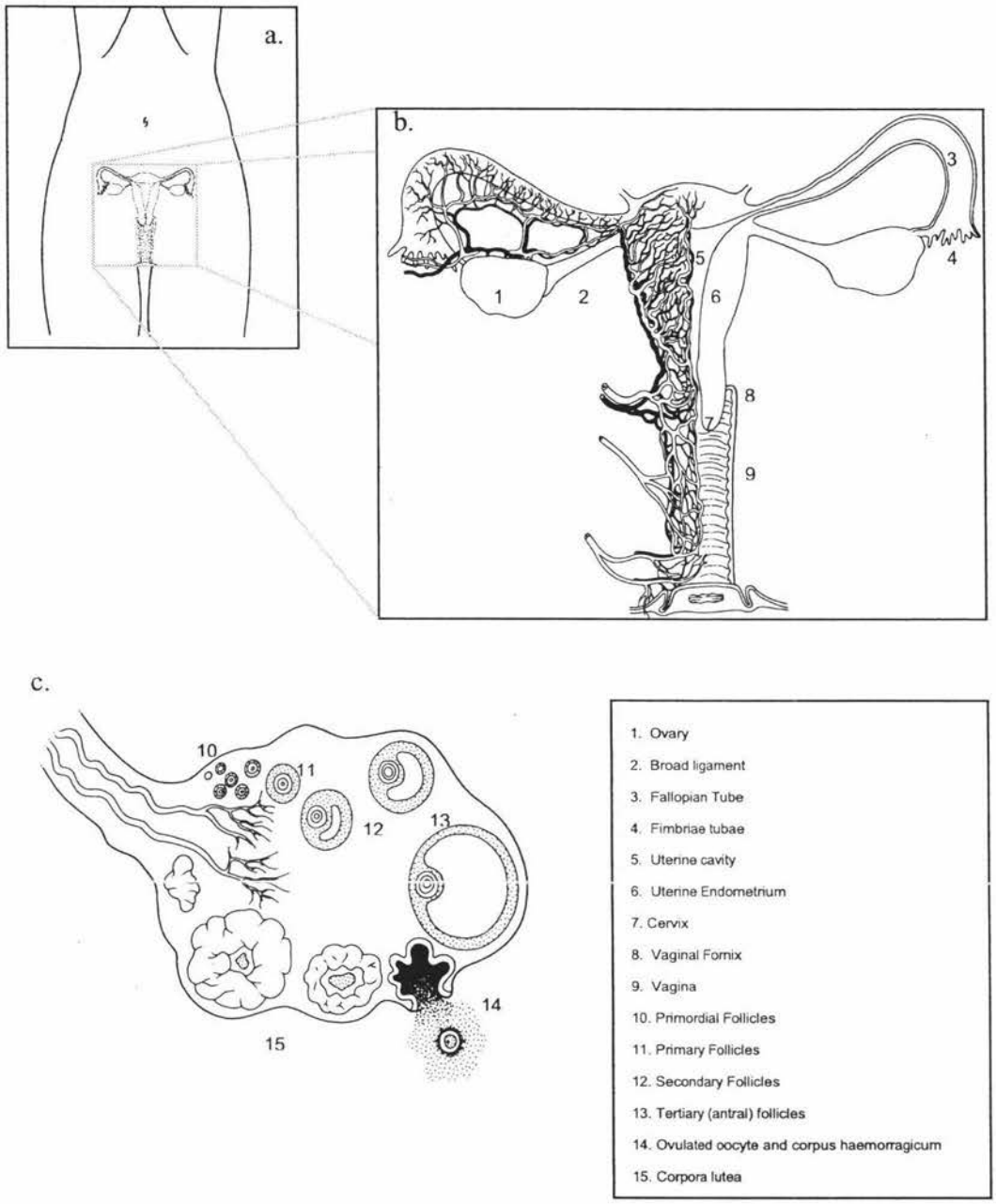


Figure 2.1. Anatomy of the human female reproductive tract*. (a) Position of human female reproductive tract in the abdomen. (b) Structure of the human female reproductive organs and genital tract, with blood supply (note that the ovarian vascularity have not been included to preserve clarity the of the diagram). (c) Structure of the human ovary, showing the phases of follicular development.

**Adapted from Cull (1989)*

Some of the primordial follicles grow to become primary follicles. They are about 100μm in diameter. The granulosa membrane is still a single layer but the cells change shape from being flat to more cuboid. The connective tissue covering the granulosa membrane begins to contain blood vessels and is called the *theca*.

Some of the primary follicles grow to become secondary follicles. In these, the granulosa cells have divided by mitosis to become 2-6 cells across the membrane. The theca is still a single layer of cells.

Some of the secondary follicles grow to become tertiary follicles. These are much larger at about 200μm in diameter. The granulosa cells have begun secreting fluid that fills the extracellular spaces and these pockets of fluid pool to form the antral space (filled with the antral, or follicular, fluid). Additional fluid passes through the theca from the blood vessels and this serous fluid augments the secretions from the granulosa cells. About 80% of the proteins found in blood are present in antral fluid (Lipner, 1973). This fluid contains proteins, steroid and protein hormones, anticoagulants (as some of the protein present is fibrin and thus there is a potential for the fluid to clot), enzymes (also some of the protein present) and electrolytes.

The theca has by now grown to form an inside (theca interna) which contains glandular cells and small blood vessels and an outside (theca externa) with connective tissue and larger blood vessels. This phase of development is heavily influenced by secretion of follicle stimulating hormone (FSH) by the pituitary gland, at the base of the brain.

Tertiary follicles are graded by their size. In humans, 1-9mm qualifies as a resting tertiary follicle, 10-14mm qualifies a ripe tertiary follicle and 15-25mm is called a Graafian follicle, after Regnier de Graaf (1641-1673). The fluid from the tertiary follicles is aspirated during IVF treatment (Hunter, 2003).

2.1.3 Structure of the oocyte and cumulus oophorus

This section provides a brief overview of the main features of the human oocyte and its associated structures. Detailed reviews of this topic are given by Crosby and Moor (1984), Crisp (1992) and Hunter (2003).

The primordial human oocyte exists in a state of arrested development, at the prophase of meiosis. At this stage it has a nucleus (known as a germinal vesicle), which contains the genetic material. The oocyte will remain in this diploid state until almost immediately before ovulation, although a number of other changes and interactions must occur before meiosis is complete.

Growth of the early follicular (granulosa) cells promotes activity in the oocyte. Most notably, the growth of an acellular layer called the *zona pellucida* (ZP). The zona pellucida can first be seen in the secondary follicle and a complete, uniform zona pellucida can be seen before the follicle reaches the graafian stage. The zona pellucida originates mainly from secretions of the growing oocyte. Areas of uniform, fine, fibrillar substances begin to appear close to the oocyte surface and develop into a mesh of interconnected filaments. As the oocyte grows, the plasma membrane (or oolemma), which surrounds the cytoplasm of the oocyte, becomes increasingly undulated. Eventually the shape of the oolemma becomes such that it forms microvilli, which project into the ZP and allow junctional contact with the extra-zonal cells, whose plasma membranes are undergoing similar changes. These gap junctions allow the transfer of material across the zona. Thus, the oocyte is not isolated from the follicular cells by the zona pellucida (Hunter, 2003).

Within the oocyte during pre-antral follicular growth there is a considerable amount of protein synthesis and accumulation of vitilline (cytoplasmic) reserves (analogous to the yolk of bird eggs). There is also an increase in cell diameter to approximately 120μm. Much of this development is directed by the action of hormones (gonadotrophin) that affect the gonads; at this stage follicular development is controlled by the action of follicle stimulating hormone (FSH), which is secreted by the pituitary gland at the base of the brain.

As the follicle develops an antral space, the oocyte is suspended in the antral fluid, held in place by a stalk of granulosa cells called the cumulus oophorus. There is a layer of cells packed closely to the oocyte within the cumulus called the *corona radiata*. The rest of the cumulus consists of granulosa cells dispersed in a gel-like matrix of hyaluronic acid. It is the coronal cells that have projected microvilli invested in the zona and are in junctional contact with the ooplasm. The cumulus oophorus contains up to 11,000 cells (Oritz & Croxatto, 1979 cited in Mohr, 1984) and measures 3-5mm in diameter (Crosby & Moor, 1984).

Prior to ovulation the oocyte resumes meiosis and, with the disintegration of the germinal vesicle, the chromosomes arrange themselves along the meiotic spindle and the process is halted following abstriction of the first polar body into the perivitillene space (refer to Figure 2.3). At about the this stage the pituitary gland releases a surge of

a gonadotrophinal hormone, leutinising hormone (LH), which causes a large amount of fluid to accumulate in one of the follicles (the dominant follicle) and approximately 36 hours after the LH surge, ovulation occurs.

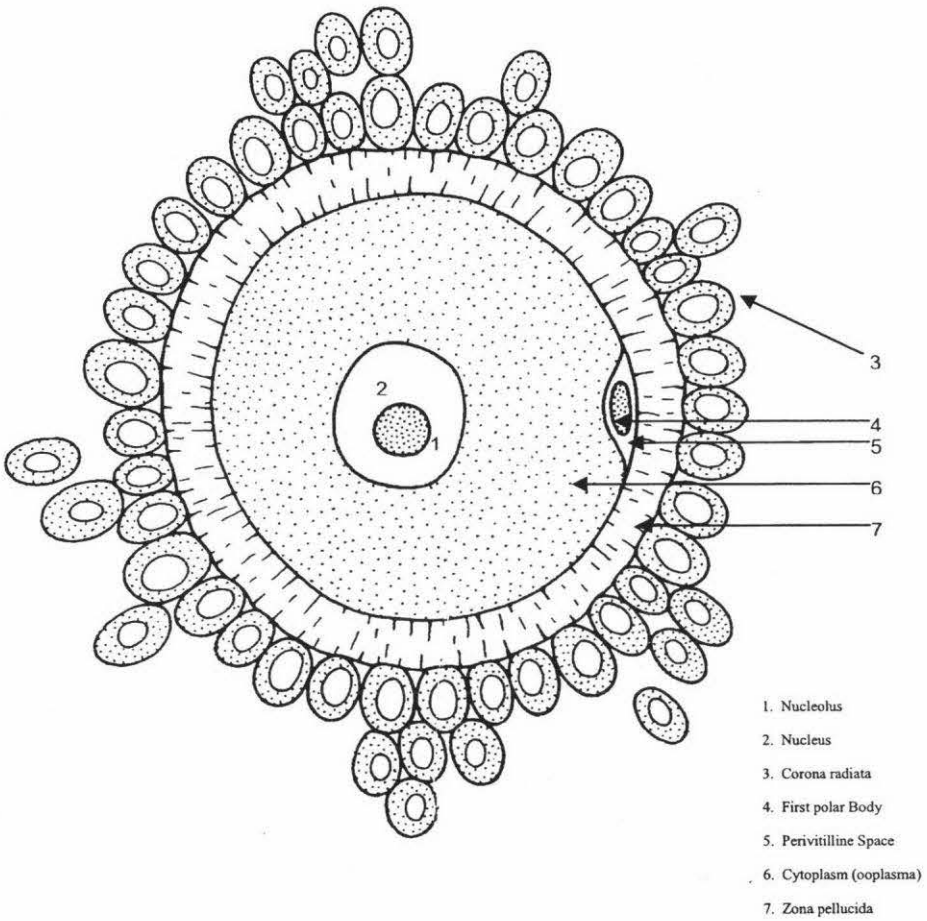


Figure 2.2 Structure of the human oocyte showing the *zona pellucida* and *corona radiata*[*].

[*]Adapted from Cull (1989)

2.1.4 Follicular fluid

What follows is a description of the liquid that fills the antral space because it is this fluid that is to be collected and analysed for dissolved oxygen levels. It is important to understand its functionality and physical properties since these will have an impact on the collection and sensing operations. Fischer, *et al.* (1992) reports the average diameter of ripe human follicles to be 16.6mm, meaning that each follicle yields but a few millilitres. It is an increasingly difficult subject of study, since work with human follicular fluid is rather sensitive from an ethical perspective, but it is also because it is

difficult to work with small sample volumes, and to sample reliably to show variation within a follicle over time. Consequently, there is much still to be understood regarding the nature of the *liquor folliculi.*

The literature relating to the properties of follicular fluid has been described in excellent reviews by Edwards (1974), by McNatty (1978) and by Gosden, *et al.* (1988).

2.1.4.1 Physical properties of follicular fluid

The liquid that inhabits the interior of the follicles is straw coloured. The yellow hue has been shown to be due to the presence of bilirubin and carotenoids (Bayer *et al.*, 1992). Spectrophotometric examination shows that follicular fluid that is free from blood has an absorbance peak at 458nm and gains several more absorbance peaks at 418nm, 540nm and 575nm as blood is introduced (Huyser *et al.*, 1993).

Rheological studies have shown that the viscosity of human follicular fluid decreases with increasing shear rate in the range 75-750s^{-1} and when exposed to a constant shear rate the viscosity falls linearly with time at a rate of 13μP min^{-1}(Luck *et al.*, 2000).

Studies on the intrafollicular fluid pressure in the rabbit have shown that it is similar to the pressure exerted on capillaries, and that it increases with increasing antral volume (Blandau & Rumery, 1963; Espey & Lipner, 1963). The significance of these findings is that the ovulatory follicle is unlikely to burst as a result of the internal pressure and is more likely to collapse as a result of structural changes in the follicular wall. The pressure is sufficient to push the liquid from the follicle slowly, as the follicle collapses. Suction is applied during aspiration of this fluid for IVF treatment because the flow of fluid is not sufficiently fast for aspiration purposes.

Observations have been made on the temperature of follicles preceding ovulation, and have shown, somewhat counter-intuitively, that the graafian follicle is slightly (but significantly) cooler than the surrounding ovarian tissue in rabbits (Grinsted *et al.*, 1980), pigs (Hunter *et al.*, 1995) and in humans (Grinsted *et al.*, 1985).

2.1.4.2 Composition of follicular fluid

Since the follicular fluid is partly a filtrate of blood plasma, it comes as little surprise that the composition is similar to plasma. The concentration of electrolytes in follicular

fluid has been measured and compared to that found in blood serum and/or plasma for various species (Gosden *et al.*, 1988). Table 2.1 shows the concentrations of electrolytes in human follicular fluid and blood plasma/serum and the normal reference blood levels.

Table 2.1. Electrolyte concentrations ($mmol.L^{-1}$) of human follicular fluid (FF) and blood plasma (P) or serum (S)*.

	Na^+		K^+	
Normal Blood Range (Anderson, 2002)	136-145		3.5-5.0	
	FF	P/S	FF	P/S
Shalgi *et al.* (1972)	124	145	4.4	4.6
Edwards (1974)	147.8	150.5	5.2	5.7
Chong, *et al.* (1977)	143	154	5.4	5.4

**Adapted from Gosden, et al. (1988)*

The protein content of follicular fluid has been investigated extensively showing that the total protein concentration is lower than in blood serum and that follicular fluid is lacking the blood proteins with higher molecular weights, due to the filtering effect of the thecal and granulosa layers. The protein composition of human follicular fluid is summarised in Table 2.2.

Table 2.2. Summary of studies into the protein content of human follicular fluid with respect to human blood serum (mg/mL).

	Total Protein		Albumin		Fibrinogen*	
Normal Blood Serum Range (Anderson, 2002)	55-80		33-55		2-4	
	FF	Serum	FF	Serum	FF	Plasma
Perloff, *et al.* (1955)	61.2	71.7	27.4	28.8		
Manarang-Pangan, *et al.* (1971)	35.8	75.7				
Shalgi, *et al.* (1973)	58	58	40	30	1.1	3.7

**values are for blood plasma.*

Shalgi, *et al.* (1973) show that the mural layers that enclose the antral fluid have a filtering effect on plasma proteins, allowing passage of about 50% of proteins with a molecular weight of 250,000 and blocking high molecular weight proteins (greater than MW=850,000) to the point where they are undetectable. They also observed that fibrinogen is blocked to a greater extent than its MW could explain, and suggested that there are further mechanisms operating, possibly similar to those that block fibrinogen from entering the cerebrospinal fluid.

2.1.4.3 Follicular fluid pO_2, pCO_2 and pH

Research into the dissolved gas compositions of human follicular fluid is in short supply. The work to date is summarised in Table 2.3. For cross-species comparison, values have also been given for porcine follicles.

Table 2.3. Summary of studies of partial pressures (mmHg) of oxygen and carbon dioxide and pH in human follicular fluid and arterial blood.

	pO_2		pCO_2		pH	
Normal Arterial Blood* Range(Anderson, 2002)	83-108		32-45		7.35-7.45	
	FF	Blood	FF	Blood	FF	Blood
Shalgi *et al.*(1972)	54.3		35.1		7.267	
Fraser *et al.*(1973)	103.5	109.8	41.7	44.1	7.344	7.306
Fischer *et al.*(1992)	59.8	102	46.9	38.3	7.33	7.41
Imoedemhe *et al.*(1993)	138.33		40.17		7.36	
Huey *et al.*(1999)	100.5		34.8		7.35	
Knudsen *et al.* (1978)	51**	41***	45**	53***	7.41**	7.36***
Gosden, R. G. & Hunter, R. H. F Unpublished, cited in Gosden *et al.* (1988)			52.1**		7.34**	
Basini *et al.*(2004)	96.0**					

**Values are normal reference ranges for adult females.* ***Values are for porcine follicular fluid.* ****Values are for porcine venous blood.*

Two of these studies show that as the size of ovarian follicles increase, the partial pressure of oxygen falls dramatically in humans (Fischer *et al.*, 1992) and in pigs (Basini *et al.*, 2004). This has been mathematically modelled, showing that the reduction in intrafollicular oxygen is due in part to an increased distance for oxygen diffusion from the follicular perimeter through the bulk of the liquid and also due to increased mural layers consuming most of the available oxygen (Gosden & Byatt-Smith, 1986).

The research of Shalgi, *et al.*(1972) describes the determination of oxygen tension in intrafollicular fluid using excised ovarian tissue. The fluid was drawn from the follicles into syringes, avoiding contamination by air and blood and the gas concentrations were determined immediately. While disruption to the gas equilibria was likely avoided after samples were drawn, there are two weaknesses in their methods. Firstly, the validity of data gathered from excised ovarian tissue has been questioned after findings that

acidosis can be induced after ischaemic periods relative to measurements on *in vivo* ovarian tissue (Knudsen *et al.*, 1978). Secondly, the construction material of the syringes is not mentioned. It is well accepted that plastics are capable of disrupting the gas equilibria in liquids as a response to gases dissolved in the polymer matrix (Scott *et al.*, 1971; Restall *et al.*, 1975; Beaulieu *et al.*, 1999; Anderson, 2002). However, the measurements of oxygen tension in follicular fluid are consistent with the studies of Fischer, *et al.* (1992) and of Knudsen, *et al.* (1978).

Fraser, *et al.* (1973) reported the oxygen tension in follicular fluid to be 103.5mm Hg and the pCO_2 to be 41.7mm Hg. It is worth noting that the investigators took measures to prevent changes to the oxygen concentration in their samples by using glass syringes with the head space filled with heparinised saline. However, they note that their measurement of follicular dissolved gases may not be valid.

The researchers who undertook the next study of follicular oxygen (Fischer *et al.*, 1992) preserved the dissolved gas concentration in the sample in a clever way. They inserted a heparinised glass capillary between the needle used to puncture the follicles and the collection tube, which was removed, inspected for the presence of an oocyte and was analysed for pO_2, pCO_2 and pH. This technique avoided any contact with air before the measurements were made and also avoided materials that may have disrupted the dissolved gas equilibrium of the follicular fluid, thus reducing errors in their measurements. Their results are comparable to those of Shagi, *et al.* (1972).

Further research was carried out, examining the effects of carbon dioxide pneumoperitoneum (a technique where the peritoneal cavity is inflated with carbon dioxide gas) for laparoscopic collection of oocytes, and the changes in follicular fluid gas concentrations (Imoedemhe *et al.*, 1993). They demonstrated that the oxygen tension of follicular fluid was 138.3mm Hg, considerably higher than previous studies, and indeed, higher than is normally available to most tissues in the body (Anderson (2002) puts normal arterial blood oxygen partial pressure in the range 83-108mm Hg). The methods used in their study may help to explain this anomaly.

Firstly, the samples of follicular fluid may have been contaminated by blood, however, the authors do not mention the degree of blood contamination that they deemed acceptable. Secondly, the fluid was collected under sterile paraffin oil, which

presumably was in equilibrium with air, in order to prevent contact with the air. The authors do not state that the paraffin oil had been pre-equilibrated with any other gas mixture and so the partial pressure of oxygen in the paraffin oil is likely to be much higher than that of the follicular samples.

The authors stated that each sample was examined for the presence of an oocyte and transported to the laboratory for dissolved oxygen determination. They show that the time lag has no significant effect on the oxygen concentration for up to 10 minutes. It is possible that in the preceding time, during examination for the absence of an oocyte, that the liquids were extensively contacted (thus increasing the rate of mass transfer between the two liquid phases) and that the two liquids were largely equilibrated before the zero time measurements were made.

Huey, *et al.* (1999) reported the partial pressure of oxygen in human follicular fluid to be 100.5 mmHg. The fluid was sampled from the collection tube at the end of the follicle aspirating needle. It has been shown that aspiration into the collection tube causes a significant rise in the oxygen concentration of the follicular fluid (Redding, unpublished data), and consequently, the quality of these measurements is questionable.

There are two observations that can be made from reviewing the literature published on the topic of dissolved gases in human follicular fluid:

- The volume of literature is extremely limited on this topic
- What has been published shows that there is still much to be understood about the dissolved gases in human follicular fluid since the few papers that have been published do not show consistent measurements.

2.1.5 Post-ovulatory events

At the time of ovulation the oocyte and follicle are separated. The follicle then fills with blood (and becomes a *corpus haemorragicum*), and blood vessels infiltrate the granulosa layers from the theca. The follicle continues to develop into a corpus luteum, and becomes a major source of progesterone, a hormone that affects the endometrial lining of the uterus. If fertilisation does not occur, the corpus luteum shrinks and loses

its functionality. In the event of fertilisation the corpus luteum produces increasing amounts of progesterone for the first weeks of pregnancy.

The oocyte, after leaving the follicle is entrained by the infundibula and guided into the fallopian tube. Contraction of the cillia inside the tube push fluid through the tubal isthmus and towards the uterus (Bellve & McDonald, 1970). At this point the oocyte is either fertilised, in which case, it will adhere to the uterine lining and develop or it will be lost amidst the menstrual material as the uterine lining breaks up. A review of implantation is given by Findlay (1984).

2.1.5.1 Post fertilisation development

This section will very briefly describe the changes that occur within the oocyte after fertilisation. This process of embryonic development is a very important part of IVF treatment, since the embryo is cultured for some time before being re-implanted. Entire volumes have been written on the subject, and such detail is not provided here. For more information see McLaren (1972) or Paulson (1997).

The ingress of a sperm cell triggers the resumption of meiosis from the metaphase II stage, ending with the extrusion of the second polar body into the perivitilline space. At this stage, there are two pronuclei, each having half the normal number of chromosomes. These two pronuclei join in a process called syngamy and form the nucleus of a cell so that the cell now has a full, diploid, set of chromosomes. Over the course of a few days the cell will cleave, still inside the zona pellucida. The cells, therefore, maintain about the same volume as the initial oocyte and sperm cells had. When the internal mass of cells has grown sufficiently, the zona ruptures and the embryo "hatches". The cells continue to divide (although any synchrony in the divisions has long been lost) and form an "outside" section of cells and a fluid filled internal cavity, which also contains a few cells. The outer layer is destined to interact with the endometrial layers and become the placenta and associated structures and the cells in the cavity develop to form the foetus.

2.2 In-vitro fertilisation

July 25, 1978 saw a revolution in human assisted reproduction technology, with the birth of a baby girl (Steptoe & Edwards, 1978). What was remarkable was that the

mother did not have functional fallopian tubes and the fertilisation occurred *in vitro* (for further reading in the history of IVF, see Biggers (1984) or O'Dowd and Philipp (1994) both of whom give fairly detailed overviews of the development of IVF).

2.2.1 The IVF process

The course of in-vitro fertilisation and zygote/embryo/blastocyst transfer is tailored to the patient. This includes selecting the course of hormone treatment, the number of oocytes harvested and fertilised, the number of embryos implanted and the fate of any spare embryos.

However, all IVF cycles involve some aspects of ovarian stimulation, oocyte harvest, oocyte culture and fertilisation and reimplantation. Each of these phases is briefly described below.

2.2.1.1 Hormone treatment

In the early days of IVF the patients' follicular cycle was allowed to run naturally, meaning that only a few oocytes could be recovered, since normally only one follicle ovulates, and there are only a few antral follicles. In order to increase the likelihood of pregnancy, procedures were developed to stimulate the ovaries to super-ovulate by administration of hormones and hormone analogues.

Most cycles now involve a pituitary down-regulation stage with a gonadotrophin releasing hormone agonist (GnRHa) such as Leuprolide or Buserelin, starting on day 21 of the menstrual cycle (Paulson & Thornton, 1997). After a period of 10-18 days the ovaries are in a resting state, since the normal follicular cycle has been halted (Paulson & Thornton, 1997). The effect of this is that there is less chance of the development of a single dominant follicle (as there is normally) and the clinician is able to time the growth of follicles for greater convenience. At this stage gonadotropins are administered, such as human menopausal gonadotrophin (hMG) or follicle stimulating hormone (FSH). Gonal F and Puregon are brands of these drugs. They are continued along with the GnRHa to prevent a premature endogenous leutinising hormone surge. When the clinician is satisfied with the status of the follicles (usually by oestradiol 17β asssay and/or ultrasonic monitoring) a dose of human chorionic gonadotrophin (hCG) is administered (such as Pregnyl). hCG mimics the effects of an endogenous LH surge and

induces the final stages of follicle development and ovulation. The subject of induction of super-ovulation has been described extensively elsewhere in the literature. For a more detailed description of hormone treatment the reader is referred to the work of Paulson and Thornton (1997).

2.2.1.2 Oocyte harvest

Approximately 36 hours after administration of hCG, ovulation will occur. This is undesirable during an IVF cycle because the oocytes will be liberated from the follicles and lost to the clinician. In order to prevent ovulation, the clinician aspirates the fluid from the follicles 34-36 hours after the hCG dose (Paulson & Thornton, 1997). It is this harvesting process that forms the focus of this work and the fluid that is collected is to have the oxygen levels characterised.

The most common method of retrieving the oocytes is by ultrasound-guided trans-vaginal aspiration. During this procedure an aspirating needle is attached to an ultrasound probe that is placed against the vaginal fornix. The ultrasound scan shows the ovary and follicles and allows the needle to be guided through the vaginal wall and into each follicle. When the needle has punctured the follicular wall, a vacuum is applied and the antral fluid (along with the oocyte and some of the mural granulosa cells) flows through the needle and into a collection tube. The aspirated fluid is examined by an embryologist for the presence of an oocyte. If the oocyte is absent from the fluid, the follicle is flushed with a liquid which is re-aspirated and frequently contains the oocyte. Oocytes are recovered from about 60-70% of aspirated follicles (Awonuga *et al.*, 1996).

The size, shape and materials of the needle and strength of vacuum have been correlated with damage to the oocyte, ZP cumulus and corona and other developmental parameters in IVF (Awonuga *et al.*, 1996; Horne *et al.*, 1996; Miller *et al.*, 2004). Generally, higher vacuums, finer needles and rougher needle interiors lead to more visible damage to the oocytes and related structures and reduced

Typically the needle is 16 gauge and a vacuum of 100mm Hg is applied, although some clinics apply vacuum using a syringe, which is unlikely to be constant or well controlled.

Prior to the use of the transvaginal technique, aspiration under laparoscopic guidance was the norm. Since this technique has been superseded, it is not described here. The reader is referred to the work of Kovacs, *et al.* (1984) for more information on this technology.

2.2.1.3 Culture and fertilisation

After the oocyte has been located in the aspirated fluid, it is allowed to further develop in an *in-vitro* environment. This environment attempts to simulate that of the interior of the human fallopian tubes in terms of temperature, dissolved gases and other dissolved substances (such as proteins, hormones, electrolytes, osmolality, pH, etc.). Optimisation of this culture environment is still largely empirical and so there are several different types of media (such as Human Tubal Fluid (HTF) and Ham's F-10). The atmospheric composition is usually 5% oxygen, 5% carbon dioxide in nitrogen and the temperature is always 37°C. Details of the materials and conditions required for one method of oocyte and embryo culture are given by Wang and Gill (2004).

The two most common methods of fertilisation are insemination and intracytoplasmic sperm injection (ICSI). Insemination, the simpler of the two methods, involves the oocyte being exposed to a number of pre-treated, viable spermatozoa, and events are allowed to take their "natural" course. ICSI is used in cases where there is a significant male factor in the infertility, and a single sperm cell is injected through the zona pellucida and oolemma into the ooplasm. ICSI is much more labour intensive. A detailed description of fertilisation techniques is given by Paulson and Francis (1997).

After fertilisation the embryos are cultured for several more days until they are discarded, frozen or implanted. This usually happens at the embryo (6-8 cell) stage anytime up to the blastocyst stage.

2.2.2 Success rates of IVF

Despite the efforts of the assisted reproduction community, the success rates are rather low (bear in mind, however, that the community deals with people for whom natural conception has failed). Table 2.4 shows some of the statistics published by the U.S. Department of Health and Human Services regarding success rates for ART in 2001.

Two features to notice are the fact that success rates decline sharply with increasing age and that the success rates overall averaged less than 30%.

Table 2.4 American ART success rates for 2001 by maternal age*.

	Maternal Age			
	<35	35-37	38-40	41-43
Number of cycles started	35984	17791	16283	7044
%Cycles resulting in pregnancy	40.6	34.4	26.2	17.3
%Cycles resulting in live birth	35.2	28.4	19.6	10.4
%Retrievals resulting in pregnancy	38.9	33.1	23.8	13.2
%Transfers resulting in live birth	41.1	35.1	25.4	14.5
Average number of embryos transferred	2.8	3.1	3.4	3.7

**Adapted from Assisted Reproductive Technology Success Rates - National Summary and Fertility Clinic Reports (2003)(Assisted Reproductive Technology Success Rates - National Summary and Fertility Clinic Reports).*

2.2.2.1 Factors affecting success rates

Clearly maternal age has a very large impact on the likelihood of birth following ART. One of the reasons for this is believed to be a reduced response to ovarian hyperstimulation. The success of ART cycles is heavily dependent on the number of embryos replaced, which in turn is dependent on the number of embryos produced and the number of oocytes obtained at aspiration. If a patient does not respond well to the gonadotropins, then fewer follicles will develop, resulting in fewer oocytes and fewer embryos and fewer high quality embryos for implantation. One method employed to counter this effect is to implant more embryos (see Table 2.4) to increase the chance of transferring a high quality embryo, which leads to pregnancy and eventually live birth. This carries an increased risk of multiple pregnancy, which can have serious implications for both the mother and the children. In New Zealand the number of embryos transfered is limited to one or two.

As noted by Shuttleworth (1909), chromosomal abnormalities become more frequent with increasing maternal age.

2.3 Prediction of oocyte developmental competence

It is evident that the development of the oocyte is triggered and directed to a certain extent by the cellular, endocrine and physical environment in which it resides. This

reasoning has led researchers to believe that certain parameters of this environment can give an indication of the developmental capacity of an oocyte resulting in myriad correlations being calculated, some stronger than others. The appeal of having a measurable parameter that correlates strongly with oocyte quality arises from the difficulty in testing an oocyte for its capacity to develop normally without impairing (or completely devastating) that capacity (bearing in mind that the oocyte is only a single cell and that its haploid state leaves it particularly susceptible to chromosomal damage).

2.3.1 Measures of oocyte/embryo quality

The first opportunity to measure oocyte quality comes after it is liberated from the follicle by microscopic examination. The presence of a germinal vesicle (and absence of the first polar body) can indicate poor quality, since the first meiotic division has not occurred, and consequently, the cell has the wrong number of chromosomes. Light microscopy can also show the state of the zona and the cumulus, which have roles to play during fertilisation, and may not function properly if damaged (Hunter, 2003).

Immediately post-fertilisation, light microscopy should show the presence of two pronuclei (one from the sperm and one from the oocyte) and not a third pronucleus, which is indicative of polyspermy (ie. penetration of more than one spermatozoan into the oocyte). Mohr (1984), provides a six point list of characteristics that can be used to grade human embryos:

- The shape and relative size of blastomers within an embryo
- The number, location, size and general appearance of nuclei within the blastomers
- The number of small anucleate cytoplasmic fragments (blebs) in the perivitilline space or between the blastomers (ie. the degree of fragmentation)
- The general appearance and distribution of cytoplasmic organelles
- Formation of junctions between the blastomers and compaction of the blastomers
- Formation of a blastocyst comprising trophoblast and inner cell mass

An emerging option for determination of embryo quality is the use of pre-implantation genetic diagnosis (PGD). This technique involves taking a cell from an embryo (about 8 cells) or blastocyst (note that several cells can be taken from the trophoderm of the blastocyst with no detrimental effects) and analysing the cell(s) for genetic aberrations. An excellent review of PGD is given by Findlay (2000). This technique falls outside of the scope of this thesis and will not be discussed further.

2.3.2 Maternal age

It was first noted in literature that with advanced maternal age came a higher incidence of "mongolism" (Down's syndrome) by Shuttleworth (1909) (cited by O'Dowd & Phillipp, 1994). Subsequently, maternal age has been shown to affect not only the "quality" of the oocytes, with respect to the number of chromosomes (trisomy 21, for example), but also the number of oocytes produced (the implications of this are described in section 2.3.3.1).

Paulson and Thornton (1997) state that the likelihood of miscarriage increases with increasing maternal age. The rate in women undergoing assisted reproductive treatment (ART) less than 40 years old is 18.5%, whereas in women over 40 years, the rate increases to 35.1%. The reasons for this trend are unclear.

2.3.3 Blood flow characteristics

It has become well accepted that the degree of vascularisation and blood flow around a follicle strongly influences the oocyte that resides inside (remembering that blood does not flow through the follicle and that the blood vessels are in the thecal layers, and do not permeate the antral space). The use of colour doppler ultrasonography has allowed relatively non-invasive imaging of bloodflow characteristics about the ovaries and the follicles therein. There is a host of literature that supports the view that follicles with better vascularisation produce oocytes that are more likely to fertilise and grow normally (Van Blerkom *et al.*, 1997; Van Blerkom, 1998; Bhal *et al.*, 1999; Coulam *et al.*, 1999; Huey *et al.*, 1999). However, the ultrasonic equipment that is capable of doppler flow imaging is costly and, because of the nature of doppler effects, is sensitive to the angle of the scanning sector relative to the direction of blood flow. A result of this is that the doppler ultrasound technique is not yet capable of giving reliable quantitative

measurements of blood velocity in a clinical setting. Bhal, *et al.* (1999) show that the amount of follicle that is surrounded by blood vessels(graded as <25%, <50%, <75% and >75%) can be correlated strongly with the likelihood of a pregnancy.

2.3.4 Follicular gas composition

It was first postulated by Gaulden (1992) that reduced dissolved follicular oxygen could impair the quality of the developing oocyte. She proposed that an hypoxic environment would induce production of excess lactate, reducing the pH of the oocyte surroundings. Such an acid environment would be capable of disrupting the microtubules that form the meiotic spindle and after a disrupted chromosomal abstriction, aneuploidy would be more likely, bringing an increased risk of abnormal embryonic development, spontaneous abortion and post natal conditions (such as trisomy 21 – Down's syndrome).

Subsequently, there have been two studies in the human attempting to relate follicular oxygen tension to oocyte developmental competence. The first was by Van Blerkom, *et al.* (1997), in which they claim that oocytes from severely hypoxic follicles were more likely to have chromosomal abnormalities than oocytes from follicles with a greater dissolved oxygen content. They found also that the ability of an oocyte to develop *in-vitro* to the metaphase II stage and to fertilise was not related to the oxygen content but that the ability to develop to the 6-8 cell stage did correlate to dissolved oxygen significantly. The measurements of oxygen in this study involved immersing an oxygen probe into the aspirated fluid after it had been collected. It has been shown that the aspiration of fluid into a vacuum-sealed collection tube significantly alters the concentration of dissolved oxygen (Redding, unpublished data). Surprisingly they do not report the actual oxygen tension in the fluids, instead, oxygen content is reported to be ≤1.5%, 1.5-2.5% or 3.0-5.5%.

The second study that attempted to correlate dissolved oxygen with developmental competence in the human oocyte was by Huey, *et al.* (1999). They show that ability to fertilise and embryo morphology does not correlate significantly with the concentration of dissolved oxygen that they measured. Again, the quality of the oxygen measurements is called into question (see section 2.1.4.3).

2.4 The product development process

The previous sections of this review have demonstrated the need for improved success rates in IVF treatments, and outlined the potential value in ovarian follicular fluid oxygen levels for providing information on oocyte quality. It is also clear that successful design of an apparatus to allow this to be done in a modern fertility clinic must consider a vast array of issues, from logistics, to clinical use, to technical feasibility.

A product development process has been adopted for this project so that there was a well defined, structured, process by which these issues were considered in order to achieve the objectives from Section 1.1:

- To develop a set of product specifications that will help to guide the design of a device for sampling follicular fluid for dissolved oxygen determination, and
- To develop a prototype device and use it in a fertility clinic

This section will describe the philosophy and purpose of the product development concept, the anatomy of a product development project and the specific stages that were anticipated to be carried out through this project.

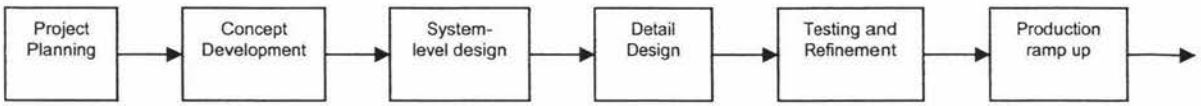
2.4.1 The philosophy of product development

The reason for the product development process is to help new products to be successful on the market. There are many variations on the theme but all of them have this goal in mind. Cooper (2001) thoroughly describes the purpose of a structured product development process, pointing out that careful planning in the early development stages is more efficient (and cheaper) than making substantial changes later in the process, as result of poor forethought.

2.4.2 Stages of the product development process

The development of new products is a process that consists of several distinct stages. The process that this project follows is that described by Ulrich and Eppinger (2000). Their process begins with the planning of the project and carries through to the production ramp-up at the end (figure 2.4).

Figure 2.4 The product development process consists of several distinct phases, each with its own objective. Adapted from Ulrich and Eppinger (2000).



2.4.2.1 Project planning

Ulrich and Eppinger {, 2000 #49} explain that this stage identifies opportunities and sorts the wheat from the chaff. The projects that appear to have merit are allocated resources and are passed on to the next phase. The planning takes into account the situation of the organisation and (especially for commercial businesses) the portfolio of developments that the organisation sees as being ideal.

2.4.2.2 Concept development

This phase is concerned with identifying what the customers want, what the product should do, how these things can be achieved and what the best way of achieving them is. This best way of achieving an end (the product concept) is passed on to the system-level design phase.

This project is concerned with the identification of the customers' needs and what the product should do. Thus, these points are expanded in more detail in section 2.4.3.

2.4.2.3 System-level design

The system-level design is concerned with sorting out the nuts and bolts of how a product will work. It may involve computer modelling/CAD designs, mathematical models of performance and working prototypes of parts of the product or even a whole working product. At this stage the designers begin to take the manufacture of the product into account and how the customer will interact with/operate the product.

When the working product has been designed and tested it is passed to the detailed design phase.

2.4.2.4 Detailed design, testing and refinement

These phases are concerned with designing the final product, how it will look, operate and perform. As the title indicates, this is an iterative process, where a product is tested, tweaked (only small changes should be required at this stage) and retested.

After the testing and refinement have been completed, then the fine details of production can be resolved and the product is released to the market.

2.4.3 Identification of customer needs and initial specifications

This phase of development begins by understanding the needs of the customers. Ulrich and Eppinger (2000)identify six distinct objectives for this exercise:

- Ensure that the product is focused on customer needs
- Identify latent or hidden needs as well as explicit needs
- Provide a fact base for justifying the product specifications
- Create an archival record of the needs activity of the development process
- Ensure that no critical customer need is missed or forgotten
- Develop a common understanding of customer needs among members of the development team

They suggest that these needs can be identified through interviews with individuals from the customer group, facilitated focus groups and observing the product in action. Griffin and Hauser (1993) discuss the various strengths and weaknesses of these methods at length.

The next stage is to take the needs that have been identified and sort them into a hierarchy of importance to the customer. The final part is to assign each need with a parameter which can be measured. The target performance of the product regarding each parameter is assigned and recorded. This is the set of initial specifications.

As stated in section 1.1 one of the objectives of this project is to form a list of initial specifications for a product that will sample follicular fluid for dissolved oxygen

analysis. The rest of this chapter is concerned with the nature of gases dissolved in follicular fluid, which influence the product design and will require appropriate product specifications.

2.5 Sampling liquids for dissolved oxygen determination (and reduction of pre-analytical errors)

Preservation of the dissolved gas equilibrium before analysis has been identified as one of the most important factors in ensuring high quality information from dissolved gas determination (Müller-Plathe, 1998). The two most important influences on dissolved gas equilibrium are the materials with which the liquid is in contact and the presence of an unequilibrated gas phase in the sample container.

2.5.1 Sampler material

It has been shown that polymeric materials can disrupt gas equilibria in liquids (Scott *et al.*, 1971; Stevens, 1992; Carignan & Gächter, 1994; Beaulieu *et al.*, 1999). Thus it is important to consider the nature of the materials used in the construction of both the sampling device and the equipment that will be used to measure the concentrations of the dissolved gases. Generally, glass is the material of choice (d'Ortho *et al.*, 2001), followed by metals (although these have the disadvantage of not being transparent) and plastics. Carignan (1994) shows that the extent to which a plastic affects the amount of dissolved gas is polymer specific, as summarised in table 2.5. This is backed up by the extensive material data available in Pauly (1999).

Table 2.5 Specific oxygen release from several different polymers into water. Adapted from Carignan & Gächter (1994).

Polymer	O_2 released ($\mu mol\ cm^{-2}$)
Teflon	0.18
Polycarbonate	0.23
Acrylic	0.10
Polyvinyl chloride	0.07
Polyformaldehyde	0.04
High Density Polyethylene	0.04
Polyvinylidene	0.05

2.5.2 Gas bubbles

Gas bubbles in liquid samples have been identified as a major problem in dissolved oxygen measurement (Mueller *et al.*, 1976; Biswas *et al.*, 1982; Pruden *et al.*, 1986; C. G. Clark *et al.*, 1998; Müller-Plathe, 1998). The best way to deal with bubbles of gas is to avoid their presence altogether.

2.6 Measurement of dissolved oxygen

A key aspect of this project is the measurement of dissolved oxygen. What follows is a description of the nature of gases dissolved in aqueous solutions and common issues associated with the measuring of dissolved oxygen.

2.6.1 Henry's law

The simplest description of the solubility of a gas is Henry's Law.

Henry's Law of dissolved gases.

$$f = k.x$$

f fugacity of gas (Pa)

k Henry's law constant (Pa)

x mole fraction of gas in solution

Henry's law states that the fugacity of a gas in solution is proportional to its mole fraction in solution. Fugacity can be interpreted as partial pressure above the solution provided that the gas behaves in a manner not too dissimilar from the ideal gas (and thus the ideal gas law). The mole fraction of gas in solution can also be interpreted as being a concentration, if the proportionality constant is adjusted appropriately. Restating Henry's law: The concentration of a gas in solution is proportional to the partial pressure of that gas above the solution. Henry's law applies to solutions where the solute particles are sufficiently dilute that there is no solute-solute interacton. It also applies only to isothermal and isobaric situations where there is no other solute dissolved in the solvent (Hitchman, 1978).

The relationship in Henry's law can be adjusted to apply to different units of concentration, other than mole fractions, by using different values (and units) of the Henry's law constant.

2.6.1.1 Temperature dependence of gas – liquid equilibria

An equation can be derived from basic thermodynamics to describe the variation of gas solubility in a solvent. The relationship is given below (Hitchman, 1978):

The relationship between solubility of a gas in a solvent and the temperature of the system.

$$\left[\frac{\partial \ln\left(x_g / x_l\right)}{\partial T}\right]_n = -\frac{\Delta H}{RT^2}$$

x_g mole fraction of gaseous component in the vapour above the solution

T	Absolute temperature (K)
R	Ideal Gas Constant (8.314 J/mol.K)
ΔH	Heat of solution (J/mol)
P	Isobaric system pressure (Pa)

This relationship holds for ideal solutions (very dilute solutions approximate ideality).

2.6.2 Applications of dissolved oxygen measurement

Once samples have been collected they must have their dissolved oxygen levels quantified. The oxygen measurement method could place constraints on the amount of sample required, or possibly other factors. To identify these potential impacts a brief overview of dissolved oxygen measurement was undertaken.

Measurement of dissolved oxygen finds applications over a variety of fields, most commonly in aqueous environmental monitoring (dissolved oxygen in rivers, streams, etc.), in wastewater treatment, in industrial fermentation operations and in clinical monitoring. Each of these applications have developed their own techniques and, with care, solutions to problems relating to dissolved oxygen monitoring in one situation can be transferred to another.

2.6.3 The Clark electrode

The current in a galvanic cell is characterised by its magnitude and direction. Thus, if the reduction of molecular oxygen was to occur at one of the electrodes (ie. the cathode), then there will be a current to that electrode.

$$O_2 + 4e^- \rightarrow 2O^{2-}$$

The amount of oxygen reduced at the electrode (w, grams) is proportional to the amount of charge (q, coulombs) that is passed from the electrode (ie. $q=it$) and is related by Faraday's law (Hitchman, 1978):

$$M_{O_2} = \frac{Mit}{nF}$$

M_{O2}	Mass of oxygen reduced at the electrode
M	molecular weight of oxygen
i	current (A)
t	time (s)
n	number of electrons per reaction
F	Faraday's constant (96,486.7C/mol)

Assuming that the rate of the reaction at the electrode obeys first order kinetics, then the rate of reaction can be expressed as:

$$\frac{dN}{dt} = k_{\mathrm{Re}\,d} C_{Ox} - k_{Ox} C_{\mathrm{Re}\,d}$$

N	number of moles of reduced species produced
k_{Red}	rate constant for the reduction reaction
k_{Ox}	rate constant for the oxidation reaction
C_{Ox}	Concentration of the oxidised species at the electrode
C_{Red}	Concentration of reduced species at the electrode

Thus, the current generated by the reaction per unit area of electrode can be expressed:

$$i = nF\left[k_{\mathrm{Re}\,d} C_{Ox} - k_{Ox} C_{\mathrm{Re}\,d}\right]$$

That is, there is a measurable current, which is proportional to the concentration of molecular oxygen at the surface of the electrode (Hitchman, 1978).

There are several major disadvantages to having an electrode in contact with a test solution. The first is that the surface of the electrode can be degraded by impurities in the environment to be tested, reducing the available area for the reaction to take place and thus reducing the measured current (causing false measurements or requiring frequent calibration). The second disadvantage is that the electrolyte concentration of the test solution may not be known or may be changing, which will affect the electrode potential, the current and thus the integrity of the measurements (which maybe be erroneous or require frequent recalibration). Thirdly, it is possible that alternative reactions could be occurring at the electrode surface and generating additional current. This would serve to inflate the measured values of oxygen in solution.

Clark first patented the membrane covered electrode in 1959 (L. C. Clark, 1959). The basis of the Clarke electrode is that there is a platinum wire that is in an electrolyte

solution of known composition and this interacts with the analyte through a membrane that is permeable to oxygen but not to other potentially detrimental species.

The reaction at the cathode is:

$$O_2 + 4H^+ + 4e^- \leftrightarrow 2H_2O$$

The anode is usually a silver/silver chloride reference electrode. The following reaction occurs:

$$Ag + Cl^- \leftrightarrow AgCl + e^-$$

The reduction of oxygen at the cathode occurs due to a potential difference applied between the cathode and the anode. This voltage allows very fast reduction at the cathode, which causes the reaction to be limited by the rate that molecular oxygen can travel from the test solution to the electrode. The distance between the electrode and the membrane is usually very small, approximately 10μm (Skoog *et al.*, 2004). Consequently, the rate of oxygen arriving at the electrode surface (and hence the current that flows through the electrode) is limited by the rate of diffusion of oxygen through the membrane, which is described below:

$$w = D.\frac{A}{x}\Delta C$$

w	rate of oxygen diffusion
D	diffusivity of membrane
A	area of membrane
X	membrane thickness
ΔC	difference in oxygen concentration across membrane

Thus it can be seen that to reduce the response time of the oxygen electrode, a material with high 'D' can be selected, a large membrane area can be used or a thin membrane can be used. Needless to say, the concentration difference of oxygen across the membrane cannot be changed since it requires either reducing the oxygen concentration in the electrolyte solution (which is reduced very quickly) or increasing the oxygen concentration in the test solution (which will directly disrupt what it is that is being measured). In practise, the thickness of the membrane is usually in the order of 20μm (Skoog *et al.*, 2004). Membranes have been made from mylar, Teflon, polyethylene,

natural rubber, silicone rubber and PVC (Hitchman, 1978). However, membranes are mostly made from polyethylene or fluoridated plastics (PTFE or FEP) as these are readily available as thin sheets, have adequate strength, the permeability doesn't change a great deal over time (an exception to this is in the presence of organic vapours) and these membranes have low crystalinity, and hence a high rate of diffusion. PTFE membranes have been made with a thickness of 0.2-1.0μm thickness (Hitchman, 1978).

The solubility and diffusivity of a gas (ie. oxygen) in a membrane can be calculated given the critical temperature and diameter of the molecule of gas are known and the structure, glass transition temperature and crystallinity of the membrane are known. Alternatively, these values can be found in published literature (see Pauly (1999) for data).

When the oxygen probe is exposed to an environment in which there is no oxygen present, there will be a small residual current flowing (although this is obviously not due to the reduction of oxygen). This current is the result of reduction of impurities in the electrolyte solution and species other than oxygen diffusing through the membrane (which isn't perfectly selective for oxygen).

The temperature dependence has been described for membrane type oxygen probes by (Briggs & Viney, 1964). As the temperature of the system increases, the current produced increases dramatically. They also identify the main source of residual current in existing oxygen probes as oxygen diffusing through the probes housing to the electrodes. They also describe several sources of loss of sensitivity and response in membrane probes as deposition of material on the membrane (they describe an accumulation of calcium carbonate whilst measuring dissolved oxygen in aerated sewage) and accumulation of suspended solids, which reduces the homogeneity of the analyte. Potentially, during the measurement of dissolved oxygen in follicular fluid protein may adsorb to the membrane surface, affecting the probe response.

It can be seen from this examination of dissolved oxygen measurement that the volume of the sample is in the order of hundreds of microlitres or millilitres. Further, some of the materials involved in oxygen measurement may have toxic effects on oocytes (such as the silver reference electrode and electrolyte) and so contact between the oocytes and oxygen measuring equipment should be avoided. Thirdly, the response time of the

equipment currently available for oxygen measurement is sufficiently slow to make in-line measurement of oxygen levels impractical, and so off-line measurements will be necessary.

2.7 Conclusions

There are several conclusions that can be drawn after an extensive review of the current literature:

- The nature of dissolved follicular gases has not been examined thoroughly and is not well described
- The role of dissolved follicular gases on oocyte developmental competence has not been well described and is poorly understood
- Measuring dissolved oxygen requires a multi-disciplinary approach to obtain samples of the highest quality and to maintain their quality

Ideally, we would like to know which embryos are more likely to lead to pregnancy, so that only one or two are implanted, reducing the chances of multiple pregnancies, while not reducing the overall chances of pregnancy, or potentially increasing it. This knowledge of the state of ART justifies the objectives set out for this project in section 1.1.

It can be seen from this review that there are three broad facets to the design of a sampling device for use in a fertility clinic. These are the clinical aspects, which are specific to the environment and personnel, the cellular and embryological aspects, involving interaction of the sampling device with the embryo and oocytes and the physical aspects, involving the interaction of the sampling device with the dissolved gas composition of the sample.

The implications of each of these aspects will be discussed in chapters 3, 4 and 5.

Chapter 3. Clinical aspects of follicular sampling

> He referred to the liquor in which the detached and ripened eggs were kept; and, leading his charges to the work tables, actually showed them how this liquor was drawn off from the test-tubes; how it was let out drop by drop on to the specially warmed slides of the microscopes; how the eggs which it contained were inspected for abnormalities, counted and transferred to a porous receptacle; how (and he took them to watch the operation) this receptacle was immersed in a warm bouillon containing free-swimming spermatozoa – at a minimum concentration of one hundred thousand per cubic centimetre, he insisted; and how, after ten minutes, the container was lifted out of the liquor and its contents re-examined; how if any of these eggs remained unfertilized, it was again immersed, and, if necessary, yet again; how the fertilized ova went back into the incubators; where the Alphas and Betas remained until definitely bottled; while the Gammas, Deltas and Epsilons were brought out again, after only thirty-six hours, to undergo Bokanovsky's Process.
>
> Brave New World, Aldous Huxley

The objective of this chapter is to provide a more insightful description of procedures at the fertility clinic. This information will in turn be used to derive product specifications that will make a follicular sampling device that is suited to the clinical environment (these are summarised in section 3.6).

This chapter is based on the author's experience at two fertility clinics, one a small clinic, the other being medium-sized. The information here is not necessarily universally applicable and inferences cannot be made with absolute certainty to other clinics, especially in countries other than New Zealand, where legislative and cultural differences come into play.

This is a structured description of "life at the clinic". It introduces the clinics, describes whom one would find there and what they do, it introduces the course of IVF treatment, the resources that are required for this treatment and finally gives a description of the oocyte retrieval procedure. In addition to ensuring that the physical specifications of sampling follicular fluid for dissolved oxygen measurement are identified, there are myriad constraints to be satisfied for use of a device in a clinical setting. The two most important considerations are that the sampling procedure should not pose a health and safety risk and that the sampling procedure should not affect the efficacy of the

treatment. An important secondary issue is the attitude of the clinical staff to such a device. It must appear to have been tested thoroughly and been carefully designed for ease of use, ease of thorough cleaning/sterilisation and must appear to serve a clearly defined purpose. When these criteria are met, clinicians will be more comfortable using a device on their patients and a trial will proceed more smoothly than if the clinicians feel unhappy about the design and operations of the device.

These specifications are identified and described in this section. Each specification in turn is given a ranking of importance, as perceived by the author. These rankings are either "imperative", meaning that the product must meet the specification for use in a fertility clinic; "recommended", indicating that the product should meet the specification and "suggested", meaning that the specification should be considered and met if the other specifications are not violated.

3.1 The clinics

Fertility Associates Hamilton serves the Waikato region of New Zealand. The clinic treats approximately 500 patients per annum, about half of whom are IVF patients. The clinic has three full time embryologists, one part time embryologist, three clinicians, one part time counsellor and approximately six nurses.

Fertility Associates Auckland serves part of the Auckland region, along with another Fertility Associates clinic located on Auckland's north shore. The clinic treats approximately 2110 patients per annum, about half of whom are IVF patients. The clinic has approximately 6 embryologists, 9 clinicians and a dozen nurses.

3.2 Personnel at the fertility clinic and their roles

There are several groups of personnel in the clinic. Each has a role to play in ensuring that the clinic operates effectively, that the patients leave with a positive experience and that (hopefully) they are pregnant. These units can be broken down broadly into the clinicians, the embryologists/laboratory staff, nurses and support staff. Each of these will be described in detail.

3.2.1 Clinicians

The clinician is a medical doctor, almost always a gynaecologist and/or obstetrician. They manage the treatment of the patient, recommend the most suitable type of fertility treatment and perform the medical procedures, such as oocyte harvest, embryo replacement and sperm aspiration. They are responsible for making the decisions that affect the patient's well-being and treatment.

3.2.2 Embryologists/laboratory staff

These are the technical and scientific experts of the clinic. The laboratory is responsible for recovering the oocytes from the follicular fluid during oocyte harvest and all the subsequent operations until they are reimplanted. These cover the culture, fertilisation (whether by exposure to sperm or by micromanipulation), examination for markers of viability or quality in the oocytes and embryos. Other laboratory operations include the freezing and thawing of "spare" oocytes and embryos, semen examinations, monitoring patient hormone levels and increasingly, pre-implantation genetic diagnosis of embryos for genetic anomalies.

3.2.3 Nurses

The nursing staff are responsible for maintaining the theatre space, materials/equipment and the general patient care and well-being. This includes the preparation before a patient arrives and cleaning up afterwards. Non-disposable equipment is cleaned according to a fixed protocol, usually a detergent wash, rinse, dry and sterilisation. The sterilisation may occur in the clinic or at an external facility (at the sterile services part of the adjoining hospital).

The nurses phlebotomise the patients for blood tests and instruct the patients on administering the hormone doses for pituitary down-regulation, ovarian stimulation and administer the final hCG dose to induce ovulation.

The nurses have a key role in the theatre assisting the clinician and monitoring the patient. During oocyte aspiration, for instance, one nurse (colloquially called the "drug nurse") administers pain relief/sedative drugs under the clinician's instruction and monitors the patient's comfort, pulse, blood pressure and oxygen status.

The other nurse (a role referred to as the "scrub nurse") aids the clinician. This nurse manages the sterile equipment and may apply vacuum for aspiration (via a syringe, otherwise suction may be applied by the clinician using a foot-switch activated vacuum pump) and passes follicular fluid to the embryologist, as well as passing information to the clinician.

3.2.4 Support staff

The support staff take care of the parts of the clinic that the others are too busy to look after. They are the receptionists who look after the patient records, timetables and bookings.

Fertility clinics also have counsellors who take care of the patient during an emotional, confusing and somewhat invasive treatment. The counsellors explain to the patient what the treatment involves and help to allay the patient's anxieties.

3.3 What happens during IVF treatment?

The treatment begins with an informal presentation, at which a nurse, a counsellor and an embryologist explain the roles that they play in the course of an IVF cycle. This allows the patients to become accustomed to the clinical environment, the people and the concepts of their treatment in a non-threatening way, made easier by the fact that they are part of a group. Sometime after the information evening, the patients have a session with the counsellor, who explains the nature of the treatment (this is necessary since the patients are usually lay-people). The counsellor also discusses how the patients may feel during the course of the treatment (including the effects of the hormone treatment).

The patients are examined by the clinician, who tries to ascertain the cause of infertility, if it is not already known. The hormone regime is selected (whether it is a long course, short course or "flare"). The pituitary down-regulation and ovarian hyper-stimulation can take up to four weeks to complete. The response of the patients to the hormone treatment is monitored by the clinician and embryologists and at the conclusion, provided that there are enough follicles, and the oestrogen level is not excessive (this is a marker of ovarian hyper-stimulation syndrome), a final human chorionic gonadotrophin (hCG) dose is administered by the nurse to induce the luteinising

hormone surge that triggers the final growth of the (now multiple) dominant follicles, oocyte maturation and ovulation. Ovulation occurs approximately 36 hours after the hCG injection.

The patient (usually accompanied by their partner) undergoes oocyte aspiration 33 hours after induction of ovulation. The oocytes are cultured for a day and fertilised. Culture continues for several more days. Alternatively, a mixture containing an oocyte and semen is injected into the fallopian tubes (Gamete Intra-Fallopian transfer, GIFT). The patients are able to visit and view the embryos, if they wish.

When the embryos are sufficiently mature (after several days' culture) the patient returns and one or two embryos are placed in the uterine cavity. The patient is given hormones to encourage the embryo(s) to implant in the uterine wall and cause a pregnancy.

Residual embryos (or oocytes) are either discarded or frozen. The clinic charges an annual subscription fee to cover the cost of maintaining the embryos (which are kept in plastic straws in liquid nitrogen) viable. Some patients request that only one or two oocytes are fertilised, often for religious reasons. Having only the required number of embryos removes the ethical dilemma of eventually discarding spare embryos.

The chance of pregnancy is increased with increasing number of replaced embryos, as is the chance of multiple pregnancy. Gestation during multiple pregnancy can have serious effects on maternal and foetal health and is discouraged in New Zealand. Fertility Associate recommend that women younger than 36 years old have single embryo replacement and two embryos replaced if the patient is older than 36. Additionally, patients younger than 36 years old, whose treatment is funded by the Ministry of Health must have only one embryo replaced. This MOH stipulation became effective on 1 January 2005.

3.4 Equipment used in the fertility clinic

The equipment used during fertility treatment is highly specialised. From the syringe used to inject the embryos into the patient to the intravaginal ultrasound probe, used to guide the aspiration needle into the follicles, to the microscope slides used to enumerate

and characterise the sperm. Furthermore, convention plays a strong part in equipment selection, since the fertility community is somewhat conservative.

3.4.1 Laboratory and laboratory equipment

The equipment used in the IVF laboratory almost always is pre-sterilised and disposable. This helps reduce the chance of contamination of the samples and transmission of disease to the patients. The laboratory uses large quantities of items such as microscopic counting chambers, embryo culture dishes, micromanipulation pipettes and petri dishes, all of which are disposable. These items come "ready to use", with no preparation required.

The consumable equipment is mostly made from polystyrene, or another clear, rigid plastic. This allows the embryologist to see the material with which they are working and to see any dirt or debris that is present on the equipment. Most of the work is done in a laminar flow hood or on a heated microscope stage.

An exception to the "ready to use-disposable" rule are glass pasteur pipettes, which are used to move embryos and extract sperm from the epididymis. These are sterilised and flame polished and/or reshaped under a butane torch immediately before use by an embryologist. This is a skilled technique that requires many hours of practice to become proficient.

The embryo culture equipment, fertilisation equipment and freeze-thaw equipment is in a separate room from the sperm handling area and office space. The embryo culture room is adjacent and linked to the theatre, where the oocyte aspiration, sperm aspiration, embryo replacement, etc occurs. On the opposite side of the theatre from the embryo culture room is the patient preparation space, also with access to the theatre.

Other laboratory equipment includes microscopes, incubators, centrifugal equipment and cryogenic dewars, all of which are standard in microbiology laboratories and all of which are impeccably clean and maintained. The dewars occupy almost all of the floor space under the benches and then some more floor space.

This is coupled with a high level of quality management in the form of monitoring the operating conditions of the equipment (temperatures, atmospheric composition in

incubators, etc.), tracking reagent/media batches. Critically, the laboratory has protocols for tracking oocytes, semen and embryos, so that they are never mismatched with the wrong patient or lost.

The laboratory space is immaculately clean, tidy and well-lit. Stock of materials, equipment and reagents are managed so that there is never a shortage. In these two clinics the laboratory space is also small, cramped and lacking spare bench space. There is seldom enough room for more that a few people to moving about without colliding.

3.4.2 Clinical/theatre equipment

The equipment used in the theatre is either disposable or amenable to extremely thorough cleaning cycles. Ideally, all items would be single-use and disposable, the reality, however, is that the cost for such a luxury would be astronomical.

The main items in the theatre are the bed, the ultrasonic probe, the patient monitoring equipment and the surface coverings, including drapes placed over the bed and patients and the aprons, gowns and coats worn by the clinical staff. The other items are syringes, plastic tubes, and regular medical paraphernalia.

The bed occupies the centre of the room. It is long enough to support the patient's torso and head. The patient's legs are elevated and supported by braces that extend from the end of the bed.

The ultrasonic equipment consists of a probe, approximately 200mm long, attached via a cable to controls, including the screen that allows the clinician to visualise the patient's ovaries. It sits at the end of the bed to one side on a trolley. It is mostly made from the characteristic beige plastic common in medical equipment. However, this lightly coloured exterior allows any dirt to be easily seen and cleaned from the surface.

When in use the ultrasonic probe is covered in a plastic sleeve that protects the transducer from the patient and vice versa. This sleeve is sterile initially and has a sterile gel applied to aid the sonic transmission through the plastic and also to improve the sonic contact with the vaginal wall.

The materials used to cover the patient during a procedure are made from cloth. Several pieces are used to cover the patient from her armpits to her toes. The clinical personnel

wear either reusable sterile cloth "scrubs" or aprons or disposable sterile plastic aprons. The hands of the clinician and scrub nurse are protected with sterile latex gloves (which, have a left and a right and are sized). The embryologists wear laboratory coats to protect themselves from accidental spills during the procedure and to add to the professional and competent image being projected to the patient and their support person. At the end of a procedure the drapes and scrubs are sent for washing and sterilisation and the disposable apparel is discarded.

The lighting in the theatre is usually bright but during procedures the lighting is reduced so that the patient feels more comfortable. Spotlights are targeted at strategic points in the room so that the clinical staff can see clearly as they are working.

The doors to the theatre from the patient preparation area are self locking, as are the doors to the embryology laboratory. The laboratory access also has a peep-hole, so that one can avoid entering at an inappropriate time. These doors lock themselves as they swing shut.

The usual medical equipment, such as collection tubes, syringes, etc., are kept on sterile paper or cloth wraps on metal trolleys that can be moved about the room. After use these wraps are also discarded and the trolleys cleaned.

The theatre also has an adjacent preparation room where the nurses are able to clean the equipment after use, store the dirty washing and store other supplies. This small room is typically cramped, being filled with baskets for the used washing, sinks, benches and shelves of supplies.

3.4.3 Other miscellany

The fertility clinic has other spaces for patient examinations, phlebotomy, a "men's room" (not to be confused with the lavatory facilities), waiting areas, storage cupboards, and a small amount of office space. A small gas cylinder bunker houses the canisters of gas that feed the incubators, usually containing 100% CO_2, 5% O_2 5% CO_2 in nitrogen (also called "tri-gas") and empty cylinders.

3.5 Oocyte pick up

The most important activity relating to this work is the oocyte pick-up (OPU). This section will describe in more detail activities from an independent observer's perspective for the benefit of future developers of products for use during this operation.

3.5.1 Preparation

Several minutes before the scheduled time for the pick-up there is a flurry of activity among the embryologists and the nurses. The embryologists load a trolley with the patient's records, and check the results from the series of hormone assays. The trolley is checked for an adequate supply of oocyte culture dishes, glass pipettes, and warmed human tubal fluid (HTF) flush medium (this is a liquid that mimics the biochemistry of the fluid that normally occupies the human fallopian tubes. It is used to flush the follicles and aspirating kit).

The nurses load their small trolleys with the collection tubes for the follicular fluid and dishes for collecting any items that have been in contact with the patient (so that they do not contaminate the clean items). The trolleys also have "hot blocks", used for maintaining the temperature of items at 37°C. The bed that occupies the centre of the room is covered in drapes, usually seconds before the patient and their partner are brought in and the patient is positioned on the bed, while the partner sits beside the patient's head so that she can be offered support during the procedure.

The clinician enters and, along with the scrub nurse, thoroughly washes their hands in a basin located nearby. The clinician and scrub nurse are assisted with donning the sterile gowns or aprons and sterile gloves. Meanwhile, the "drug" nurse attaches the patient to the equipment for monitoring her pulse, blood pressure and oxyhaemoglobin saturation and places the drapes over her.

The clinician introduces the patient to the people present, enquires about the hormone assays and helps the embryologist or nurse fit the sterile sleeve on the sonic transducer (this requires some skill, given that part of it is sterile, part is non-sterile and nobody should touch both of these parts). The lights are dimmed and background music is played.

The clinician instructs the "drug" nurse to administer pain relief, specifying the drug and dose. The drugs, which are kept in a locked cupboard nearby, are brought out and after the nurse has explained that this will hurt a little are administered. The embryologist opens the outer envelope of the oocyte aspiration kit so that the scrub nurse can take and open the inner pack, and retrieve the sterile kit inside. The scrub nurse removes the protective plastic cap from the end of the aspiration needle and passes it to the clinician, who places the needle through the guide clipped to the side of the ultrasound probe.

3.5.2 The aspiration procedure

When the patient is comfortable the doctor places the ultrasonic probe in the vaginal fornix, visualises both ovaries and the antral follicles and, after explaining that there will be a sharp pain, pierces the vaginal wall with the aspirating needle and pushes the tip of the needle into a follicle.

At this point the clinician gives a signal and the scrub nurse pulls back the plunger on the syringe connected to the aspiration kit or activtes a vacuum pump and follicular fluid flows into the collection tube. The screen of the ultrasound probe shows the follicle collapsing about the tip of the needle and the surrounding follicles. As the flow slows the nurse alerts the clinician and when the flow has stopped or when the collection tube is full, they disconnect the collection tube, replace it with an empty tube and place the fluid-bearing tube in a hot block for the embryologist to examine. The clinician pushes the tip of the needle into an adjacent follicle and the process continues.

The embryologist takes note of the number of follicles aspirated, the number of oocytes recovered and the time taken for the procedure. The tubes of follicular fluid are poured into a petri dish on the microscope stage, so that it forms a relatively shallow film of liquid. In this film the oocytes and the cumuli oophori are easy to find. As each oocyte is found the embryologist informs the clinician. It is then removed from the follicular fluid using a pasteur pipette and placed in warm culture medium. Each oocyte is washed several times in culture medium before finally being placed in a culture dish filled with medium. The follicular fluid is retained until the end of the procedure.

The clinician can opt to flush the collapsed follicles to increase the chance of recovering oocytes. This involves injecting the flush medium back through the aspirating needle.

In the case of a double lumen needle there is a separate channel for this (figure 2.3) or in the case of a single lumen needle the collection tube is removed and the flush medium is injected back into the PTFE tubing.

After the first ovary has had all of its follicles drained the clinician moves to the second ovary and the process begins again. The patient may be given more medication at any stage, under instruction from the doctor. It is undesirable to remove the needle from the ovary once it has been inserted as this causes serious discomfort to the patient. As the procedure continues the aspirated fluid becomes increasingly blood stained as each punctured follicle begins to bleed. Some of the fluid can be heavily blood stained. This is also notable at the beginning and end of aspiration of each follicle.

Occasionally the follicular fluid may clot inside the aspiration kit. In this case the needle is withdrawn from the ovary, flushed with HTF medium to retrieve any oocytes held insde the kit and replaced back into the ovary.

3.5.3 Physical dimensions

In such a pivotal position, size does matter. It is suggested that a sampling device be small enough to fit in the hand of the operator. Anything larger is going to have a serious impact on the aspiration procedure. It is also suggested that a sampling device weigh less than 0.4kg.

3.5.4 Usage

The number of people available to work in this confined space is limited. Thus, it is recommended that a sampling device be able to be operated by one person only, who is also supporting its weight (since there are no flat or stable surfaces available). This is further complicated by the fact that this person must have "scrubbed up". It is imperative that a sampling device be capable of being operated aseptically. The operator will be using the device for the length of time for an oocyte pick up, approximately 20 minutes, and so it is suggested that comfort during use be considered in the design.

3.5.5 Sampler retention volume

The volume of the sampling apparatus should be as small as possible. Increasing the volume of the entire system has been shown to reduce the effectiveness of the applied vacuum used to aspirate the follicle (Horne *et al.*, 1996). Further, the clinical team involved in this study expressed concerns about the volume of the sampler and the impact that delaying the examination of a follicle's fluid for the presence of an oocyte would have on the decision to flush a follicle after the initial aspiration. An increase in volume of 2ml of the aspiration kit begins to approach the volume of an entire small follicle.

It is acceptable to increase the volume of the standard aspiration kit by 1.5ml (recommended). This is smaller than most follicles.

3.5.6 Sample volume

The volume of the sample should be as small as possible. The clinicians involved in this study had serious concerns regarding the sample size. There is a conflict between the ease of handling a larger sample and avoiding disruption to the aspiration process by taking a small sample. A minimal sample volume must be sufficient to meet the requirements of the instrument that will be used to measure the dissolved oxygen. Generally, clinical gas analysers require less than 300µl samples for dissolved oxygen, dissolved carbon dioxide and pH measurements. The clinicians were satisfied that a sample volume up to 600µl would not severely impact on their operations. It is suggested that this constraint be met by a sampling device.

3.5.7 Post procedure activity

After the procedure the patient is left to relax for a few minutes. Meanwhile the embryologist clears up the used petri dishes and collection tubes that have accumulated on the trolley and takes the trolley back into the embryo culture room. The embryologist re-screens the retained follicular fluids for oocytes and places all the oocytes, in culture dishes in an incubator. The trolley and microscope stage are cleaned with 70% ethanol solution.

The scrub nurse clears flushes the aspiration kit one final time and collects this fluid for the embryologist to examine. All sharp items are disposed in a "sharps" receptacle and

all other rubbish is disposed of into biohazard bins. The plastic is removed from the ultrasound probe and the bed is stripped down. The reusable items are taken to the adjacent scrub room for cleaning.

The room must be cleared and cleaned swiftly, since it is required for use frequently, often with back-to-back bookings.

Finally, the patient is taken to another room to recover from the effects of the sedatives.

The whole procedure takes approximately 20 minutes

3.5.8 Time delay before measurement

Studies into preanalytical errors in blood gas measurement find that the length of time between sampling and measurement can affect the quality and reliability of the measurement. The length of time will determine the extent to which the liquid sample will equilibrate with gas bubbles in the sample and the sampler materials, thus the longer the delay, the less likely the measurement reflects the true gas content of the samples. These time related effects need to be balanced against the other responsibilities of the embryologist who is likely to make the measurement after the oocyte pick up. This delay between the oocyte aspiration and the dissolved oxygen determination may be up to 20 minutes.

Cellular effects can also proceed with time. It has been recommended that blood samples in plastic containers be measured within 15 minutes (Müller-Plathe, 1998). Follicular fluid does not have the same oxygen buffering capacity of blood and so lowering the temperature will have subtle effects. If the sample is refrigerated, measurement should be immediate. If the measurement cannot be made within 15 minutes, a glass container should be used and the sample held in ice water for no more than one hour (Müller-Plathe, 1998).

3.5.9 Sample storage temperature and cellular consumption of oxygen

Cellular glycolysis continues after sampling blood for measurement of gases and metabolites. This metabolic activity causes a reduction of pH, HCO_3^- and pO_2 and increases pCO_2. This activity can be reduced substantially by cooling the sample to

approximately 4°C (immersion in ice water in practice). The reduction in dissolved oxygen is caused mainly by the oxidative action of leucocytes and platelets.

Samples of follicular fluid are unlikely to contain substantial numbers of leucocytes or platelets, and so the effect of cellular activity on dissolved oxygen is unlikely to be important and the samples do not need to be stored at reduced temperatures. Additionally, reducing the temperature will decrease Henry's law constant for the follicular sample. The effect of decreasing Henry's law constant is that the rate of oxygen transfer from any bubbles and/or plastics will increase (due to the increased partial pressure gradient between the materials and the increased deviation from the equilibrium described by Henry's law for dissolved gases).

These guidelines (section 3.5.8) for treatment of samples for blood gas analysis may be applied to samples of follicular fluid as a minimum standard, follicular fluid needing more care than blood, since follicular fluid does not contain haemoglobin to buffer changes in oxygen tension.

Despite the reasons for not chilling the follicular samples given in this section, it is possible, even likely, that some samples will get treated in this manner. Thus, a sampling device must be able to handle these conditions.

The problem is now twofold. Firstly, the sampling device and sample container must be able to withstand a rapid change in temperature from ambient (or even 37°C) to 0°C without a detrimental effect on the sample or the integrity of the container that holds the sample. The sampling device and container must meet this criterion.

Secondly, the sampling device must be able to withstand exposure to water, since a sample on ice will be exposed to the molten product of the chilling medium or condensed water from the atmosphere. It is suggested that that this specification also be considered during design.

After discussions with the clinical staff it was established that they would the loss of information for a follicular sampling device for 1 in 50 samples, or 2%.

3.5.10 Tracing the source of a sample

In order to gain useful and relevant information about the quality of an oocyte, the clinician must be certain that the sample of follicular fluid can be linked to a particular follicle and oocyte. This raises several issues.

Firstly, the extent of mixing of fluids from different follicles within the aspiration kit and sampling device must be minimal prior to dissolved oxygen determination otherwise the oxygen content of the fluid may be changed. A sample for dissolved oxygen analysis should contain at least 95% of one known follicle.

Secondly, it is imperative that the oocyte from a particular follicle be traceable to a particular sample of follicular fluid. This issue has two parts. The first is knowing where an oocyte came from and where the corresponding follicular fluid is and the second is recording this information for later retrieval, not just to the patient level but to the ovary, follicle and oocyte. This should be achievable with the loss of information of one sample in 50 (based on the discussions with clinical staff).

3.6 Summary of specifications

The specifications for a follicular sampling device described in this chapter are shaped by the environment in which it would be used, the people who would use it and the values that are important to them. The need statements and the design aspect to which they relate are summarised in Table 3.1 and the specifications in table 3.2.

Table 3.1 Summary of clinical need statements for a follicular sampling device.

Need Ref	Section Ref	Need Statement	Rank*
		Sampler module	
3.5.3a	3.5.3	Sampler is not too large	S
3.5.3b	3.5.3	Sampler is not too heavy	S
3.5.4a	3.5.4	Sampler can be operated in confined space	S
3.5.4b	3.5.4	Sampler can be operated comfortably for more than 20 minutes	S
3.5.10a	3.5.10	A sample consists of fluid from one follicle only	I
3.5.5	3.5.5	Sampler does not increase aspiration kit volume	R
		Sample container module	
3.5.8a	3.5.8	Sample container can contain a sample for an adequate length of time	R
3.5.9a	3.5.9	Sampler and sample container can tolerate temperature drop from 37°C to 0°C	S
3.5.9b	3.5.9	Sampler and sample container can tolerate being immersed in water	S
3.5.8b	3.5.8	DO can be measured quickly	R
		Oxygen measurement module	
3.5.6	3.5.6	Sample volume is small	S
		Procedure module	
3.5.10b	3.5.10	A sample must be traceable to an oocyte	I
3.5.10c	3.5.10	A sample must be traceable to a follicular fluid	I

3.5.10d	3.5.10	A sample must be traceable to a patient	I
3.5.10e	3.5.10	The sample source information can be recorded	I

*I – Imperative; R – Recommended; S – Suggested.

Table 3.2 Summary of clinical specifications

Spec. Ref	Need Refs	Metric	Unit	Value
Sp1	3.5.5	Sampler volume	ml	<1.5
Sp2	3.5.6	Volume of liquid removed	µl	<600
Sp3	3.5.3a 3.5.3b	Size of sampler	mm	120x70x30
Sp4	3.5.4a 3.5.4b	Number of people required to operate sampler	#	≤1
Sp5	3.5.8a 3.5.8b	Length of time from sample collection to end of oxygen determination	minutes	<60
Sp6	3.5.9a 3.5.9b	Temperature range sampler and sample container can tolerate	°C	<0-37
Sp7	3.5.9	Sampler and sample container can tolerate being immersed in water	Y/N	Y
Sp8	3.5.10a 3.5.10b 3.5.10c 3.5.10d	Number of times information regarding the source of a follicualr sample is lost	%	<2

3.7 Conclusions

The clinical requirements for a follicular sampling device are obviously numerous. It is a multifaceted problem that requires careful planning through the design and implementation.

What remains to be seen, however, is whether all these specifications can be met and a working device developed that can answer the question "Does dissolved follicular oxygen predict oocyte developmental competence?"

Chapter 4. Cytological constraints on sampling devices

Neutrals tread on eggs but break none.

German proverb

This chapter deals with the aspects of a follicular sampling device that affect the oocyte, cumulus and the resulting embryos. Oocytes by nature are fragile, both physically and physiologically, as a result of their large physical size and haploid state. Thus, special attention should be paid to these aspects of a follicular sampling device.

4.1 Minimising effects to the oocyte – cumulus complex (OCC)

The sampling procedure must not damage the oocyte or the cumulus oophorus. This also includes preventing the loss of an oocyte. A device that damaged or lost oocytes is extremely unlikely to be desirable to the clinicians of a fertility clinic, since, as we have seen, the likelihood of a pregnancy and live birth is heavily dependent on a good supply of high quality oocytes and the resulting high quality embryos.

4.1.1 Minimise effect of shear forces on OCC

In preparation for intracytoplasmic sperm injection (ICSI), an oocyte is cultured with its cumulus intact, after which, the cumulus is stripped away from the zona pellucida using a combination of enzymatic degradation (exposure to hyaluronidase) and shear forces (the oocyte-cumulus is repeatedly sucked through a very narrow channel). For more information about fertilisation techniques see Paulson and Francis (1997).

Excessive shear on an OCC before the culturing process are very undesirable. The cumulus is left intact for the culture period and is left intact for insemination (since the cumulus interacts with the spermatozoa). Thus, a damaged OCC will impact on the culture process. Excessive shearing forces on an OCC would appear as a deformed or denuded cumulus, cracked zona pellucida, and in extreme cases, complete lysis of the oocyte.

A device for sampling follicular fluid should never destroy the oocyte. Slight deformation of the cumulus is acceptable, but removal is not. The zona should not be damaged.

Clearly, a developer cannot aspirate an infinite number of test oocytes to ensure these criteria are met and so some more sensible numbers can be specified. It is acceptable to deform the cumulus of 10% of aspirated oocytes, since this is unlikely to have a serious effect on the developmental potential of the oocyte (recommended). It is acceptable to partially remove the cumulus from 1% of aspirated oocytes. This specification is "recommended". Less than 0.2% of aspirated oocytes should have a broken zona, have suffered cell lysis or have lost the majority of the cumulus oophorus. It is imperative that the sampling process does not damage the zona pellucida, lyse the oocyte or completely strip the cumulus oophorus.

4.1.2 Minimise temperature effects

As the follicular fluid is drawn into the sampling device there may be a temperature gradient between the liquid (37°C) and the interior walls of the device. A resulting temperature drop could have detrimental effects on an oocyte that was drawn into the follicular fluid sample. This temperature drop could be sufficient to cool the oocyte and depolymerise any metaphase spindle fibres that existed in the ooplasm (Moor & Crosby, 1985; Pickering *et al.*, 1990; Wang *et al.*, 2001). Alternatively, the sampling device may be warmer than 37°C, in which case an entrapped oocyte could be exposed to warmer conditions, causing increased cellular activity and possibly protein denaturation, an effect which is highly undesirable.

Thus, to ensure that an entrapped oocyte is not exposed to a temperature drift, it is recommended that the sampling device does not allow the temperature to drop below 33°C and imperative that the temperature does not rise above 39°C until the sample has been screened for an oocyte. Temperature increases have a more serious effect on cell viability, since there will be protein denaturation, compared with cooling, which causes depolymerisation of the meiotic spindles. The depolymerisation is reversed when the temperature is restored to 37°C.

After it has been screened the maintenance of a constant temperature is less important.

4.1.3 Loss of oocyte

The loss of an oocyte is unacceptable. It negates the need to measure the environment from which it originated in order to know its quality, it also seriously undermines the efficacy of the fertility treatment.

It is imperative that a sample can be examined by an embryologist for an oocyte in less than 30 seconds so that entrapped oocytes can be recovered.

A device for sampling follicular fluid should allow a trapped oocyte to be recovered after it has been located, it is imperative that this takes an embryologist less than 15 seconds.

Furthermore, it is recommended that less than 1 follicular sample in 100 (1%) should contain an oocyte and it is imperative that less than 1 in 500 samples cause an oocyte to be completely lost.

These criteria do not negate the previous specifications regarding damage to an oocyte, so that if an oocyte is caught in the sample, it still suffers no damage.

4.2 Minimise embryotoxicity of materials

While prevention of mechanical damage to the oocytes greatly reduces the risk of affecting the efficacy of the treatment, the oocytes are still at risk from embryotoxic compounds that may be among the sampler materials. There are a large number of materials that have embryotoxic and/or teratogenic properties, making this stage of design additionally important. In order to minimise the risk of using materials that could have toxic effects the equipment underwent three stages of hazard management.

4.2.1 Materials selection

In an effort to reduce the risk to the oocytes and embryos the materials used should be carefully selected. The author recommends that the materials from which the sampling device is constructed, and especially those parts of the sampling device that would contact the flow of follicular fluid, the oocyte-cumulus complex and the follicular sample should be constructed from materials that are not known to be cytotoxic, embryo-toxic or teratogenic.

4.2.2 Materials preparation/cleaning

Despite the design of a device using selected materials that had a low risk of having a toxic effect on the oocytes, the equipment is likely to be washed according to an exisiting protocol for materials that are likely to come into contact with oocytes or embryos at the clinic in which it is used. An example protocol involves a four hour soak in 5% detergent solution (for example, Pyroneg, DiverseyLever New Zealand Ltd., Auckland, New Zealand) followed by six rinses in de-ionised water (milliQ treated water, for instance). After rinsing, the items would be dried in hot air and sterilised.

It is recommended that a follicular sampling device should withstand this treatment through at least 100 cycles without suffering any damage.

4.2.3 Embryotoxicity testing

Embryotoxicity testing (Ackerman *et al.*, 1984) uses an animal model to predict harmful conditions for human embryos. The technique uses mouse embyros, usually at the 2-cell stage, and contacts the embryos with the test material. The proportion of embryos that develop to a blastocyst several days later should be greater than 80% of the proportion of control embryos (that is, mouse embryos that have not been exposed to the test material) that develop to blastocyst.

Following the cleaning protocol, the materials of construction for the sampling device should pass the embryo-toxicity screen. This is imperative.

4.3 Summary of specifications

The need statements developed in this chapter relating to the biological impacts of sampling follicular fluid are summarised in Table 4.1 and the specification is Table 4.2.

Table 4.1 Summary of need statements relating to cytological effects of sampling follicular fluid and how they fit into the modular architecture of the sampling system.

Need Ref	Section Ref	Need statement	Rank
		Sampler module	
4.1.1a	4.1.1	Sampling does not deform the cumulus oophorus	R
4.1.1b	4.1.1	Sampling does not break the cumulus oophorus	R
4.1.1c	4.1.1	Sampling does not strip the cumulus	I
4.1.1d	4.1.1	Sampling does not damage the zona pellucida	I
4.1.1e	4.1.1	Sampling does not damage the oocyte	I
4.2.1a	4.2.1	Sampler materials are not known to be cytotoxic	R
4.2.1b	4.2.1	Sampler materials are not known to be embryotoxic	R
4.2.1c	4.2.1	Sampler materials are not known to be teratogenic	R
4.2.2a	4.2.2	Sampler can tolerate wash cycle	R

4.2.3a	4.2.3	Sampler passes mouse embryo testing	I
4.3a	4.3	Sampler tolerates sterilisation by steam	R
		Sample container module	
4.1.2a	4.1.2	Sampling does not raise the temperature	I
4.1.2b	4.1.2	Sampling does not reduce the temperature	R
4.1.3a	4.1.3	A sample can be screened for the presence of an oocyte	I
4.1.3b	4.1.3	An oocyte can be recovered from a sample	I
4.1.3c	4.1.3	A sample does not contain an oocyte	R
4.1.3d	4.1.3	The oocyte is not lost	R
4.2.1d	4.2.1	Sample container materials are not known to be cytotoxic	R
4.2.1e	4.2.1	Sample container materials are not known to be embryotoxic	R
4.2.1f	4.2.1	Sample container materials are not known to be teratogenic	R
4.2.2b	4.2.2	Sample container can tolerate wash cycle	R
4.2.3b	4.2.3	Sample container passes mouse embryo testing	I
4.3b	4.3	Sample container tolerates sterilisation by steam	R

Table 4.2 Summary of specifications relating to cytological impacts of sampling follicular fluid

Spec Ref	Need Refs	Metric	Unit	Value
Sp9	4.1.1a 4.1.1b 4.1.1c 4.1.1d 4.1.1e	Rate of OCCs showing shear effects	%	<2
Sp10	4.1.2a 4.1.2b	Temperature of sample	°C	33-39
Sp11	4.1.3a	Length of time required to find an oocyte in a sample	s	>30
Sp12	4.1.3b	Length of time required to recover an oocyte from a sample	s	>15
Sp13	4.2.1a 4.2.1d	Construction materials are not cytotoxic	Y/N	N
Sp14	4.2.1b 4.2.1e 4.2.3a 4.2.3b	Construction materials mouse embryo testing	Y/N	Y
Sp15	4.2.1c 4.2.1f	Construction materials are not teratogenic	Y/N	N
Sp16	4.2.2a 4.2.2b 4.3a 4.3b	Number of wash/sterilisation cycles until sampler or sample container fail	#	>100

4.4 Conclusions

It can be seen through this chapter that the application for this sampling system and the necessity not to harm the oocytes adds a new dimension to the design of the sampling system which may not be applicable in other fields where sampling for dissolved oxygen determination is routinely carried out.

However, the dissolved gas equilibrium itself must also be taken into account, since this also is vulnerable to disruption.

Chapter 5. Physical aspects of measuring dissolved intrafollicular oxygen

All science is either physics or stamp collecting

Ernest Rutherford (1871-1937)

This section deals with the issues involved in taking a small sample of follicular fluid for dissolved oxygen determination without disrupting the dissolved gas equilibrium. The nature of the issues addressed in this chapter have been grouped together because they are physical effects that will be common in any situation where such a liquid is being sampled for dissolved oxygen determination, and not just those constraining factors that are linked to working in an assisted reproduction clinic (these specific clinical issues are addressed in Chapter 3). Each of these issues will be examined in some detail and quantifiable product specifications will be identified.

5.1 Minimisation of preanalytical errors in follicular samples

A major source of error in blood gas analysis is introduced in the pre-analytical stages, that is after the liquid is sampled and before the measurements are made (C. G. Clark *et al.*, 1998). Any measures identified that will improve the accuracy of blood gas analysis could offer benefits in the sampling and measurement of dissolved oxygen in follicular fluid. The key recommendations that have been made through a host of published literature involve the proper selection of materials for sampler construction, the effect of gas (air) bubbles, time delay between sampling and measurement and the storage temperature before measurement.

5.1.1 Blood contamination

Follicular fluid is largely acellular, and normally it contains no blood or haemoglobin. However, during the collection of oocytes blood can be introduced into the liquid as the aspirating needle pierces the vascular thecae, rupturing the local vessels and aspirating an amount of blood with the follicular fluid. The extent of blood contamination ranges from undetectable to heavy. The extent of blood contamination is best judged using an

experienced pair of eyes (Levay *et al.*, 1997), a method that has been shown to be better than spectrophotometric methods, haematocrit determination and detection using urinalysis dipsticks.

The literature describing the effect of mixing whole blood with another liquid on the dissolved oxygen of the mixture is non-existent. However, if valid measurements of follicular dissolved oxygen are to be made then contamination by blood needs to be taken into consideration, and can be ignored later if it is found to be minimal. This effect was calculated and summarised below. The detailed calculations are given in appendix 1.

5.1.1.1 System description

The purpose of this model is to predict the effect on the dissolved oxygen in the follicular fluid of mixing a relatively small amount of blood with a relatively large volume of follicular fluid.

Initially there are two separate liquids. One is largely the same as blood plasma (follicular fluid) and is thought to have a lower oxygen tension than blood (Gosden & Byatt-Smith, 1986) and obeys Henry's law of dissolved gases. The other liquid, whole blood, contains an amount of plasma, and an amount of red blood cells which contain haemoglobin. The haemoglobin will have an amount of oxygen bound (oxyhaemoglobin) that is dependent on the oxygen tension of the blood and can be predicted (as a proportion of oxygenated vs. total haemoglobin) by the Hill equation (eq. 3.1). This is discussed by Fournier (1999) although other relationships have been described to account for changes in temperature and equilibrium oxygen partial pressure (Severinghaus, 1979).

The Hill equation relates the partial pressure of oxygen to the extent of haemoglobin saturation.

$$\%saturation = \frac{Po_2{}^n}{P_{50}^n + Po_2{}^n}$$

Po_2 Partial pressure of oxygen (mmHg)

P_{50} Partial pressure at which 50% of the haemoglobin exists as oxyhaemoglobin (mmHg)

n Hill equation constant

5.1.1.2 Formulation

An oxygen balance was applied over the mixing of two liquids taking into account the oxygen in the follicular fluid, blood plasma and haemoglobin, and after mixing and the redistribution of oxygen between that dissolved in the liquid and that bound to haemoglobin. The formulation makes a number of assumptions:

- The oxy-haemoglobin dissociation curve (described by the Hill equation) does not change. Factors that do affect the dissociation curve include temperature, pH, and 2,3-diphosphoglyceric acid (DPG) concentration (negligible red blood cell lysis)
- There is no exchange of material with the environment (especially with regard to water, oxygen and haemoglobin)
- Follicular fluid obeys Henry's law of dissolved gases with respect to oxygen (this is very likely since, according to Fournier (1999), blood obeys Henry's law)
- Henry's law constant for follicular fluid is the same for that of plasma (refer to the similarity between the composition of plasma and follicular fluid, section 2.1.4.2)
- The pressure of the system remains constant
- The red blood cells (and so the haemoglobin) are distributed throughout the liquid phase homogeneously (ie. the liquid is well mixed)
- Equilibrium is reached

A word equation for an oxygen balance yields:

$$O_2 \text{ in FF} + O_2 \text{ in blood} = O_2 \text{ in mixture}$$

By considering the biphasic presence of oxygen in the blood, this can be expanded to give:

$$O_2 \text{ in FF} + (O_2 \text{ bound to haemoglobin} + O_2 \text{ in plasma}) = O_2 \text{ in (FF + plasma)} + O_2 \text{ bound to haemoglobin}$$

Yielding the equation:

$$\left(\frac{Po_{2FF}.V_{FF}}{H_{FF}}\right)+\left[\left(\frac{Po_{2Blood}}{H_{Blood}}(1-Hct)V_{Blood}\right)+\left(cHb_{Blood}.\frac{Po_{2Blood}^{n}}{P_{50}^{n}+Po_{2Blood}^{n}}.(8\times16)\right)\right]=$$

$$\left[\frac{Po_{2mix}}{H_{mix}}.\left(V_{FF}+(1-Hct)V_{Blood}\right)\right]+\left(cHb_{mix}.\frac{Po_{2mix}^{n}}{P_{50}^{n}+Po_{2mix}^{n}}.(8\times16)\right)$$

With the following nomenclature:

Variable	Symbol	Unit
Henry's Law constant for follicular fluid	H_{FF}	mmHg.mL/g
Henry's Law Constant for Plasma	H_{Blood}	mmHg.mL/g
Henry's Law Constant for a mixture of blood and follicular fluid	H_{Mix}	mmHg.mL/g
Volume of follicular fluid added	V_{FF}	mL
Volume of blood added	V_{Blood}	mL
Total volume of blood and follicular fluid	V_{Mix}	mL
Partial pressure of oxygen in follicular fluid	$Po_{2\,FF}$	mmHg
Partial pressure of oxygen in blood	$Po_{2\,Blood}$	mmHg
Partial pressure of oxygen in blood-follicular fluid mixture	$Po_{2\,mix}$	mmHg
Concentration of haemoglobin in blood	cHb_{Blood}	mmol/L
Concentration of haemoglobin in final mixture	cHb_{mix}	mmol/L
Volumetric proportion of whole blood occupied by red cells (haematocrit)	*Hct*	%
Partial pressure of oxygen at which 50% of haemoglobin is saturated with oxygen	P_{50}	mmHg
Hill equation constant	*n*	-

This equation cannot be arranged to give an explicit expression for $Po_{2\,mix}$ and so it was solved as a problem of minimisation of the difference between the LHS and RHS as a function of $Po_{2\,mix}$ in the MATLAB Version 5.3 release 11 (The Mathworks, Inc., MA, USA) environment. The MATLAB m-files are given in Appendix 1.

The values for the input parameters for the solution of this problem were taken from the literature:

Quantity	Unit	Value	Source
H_{FF}	mmHg.mL/g	23125000	Fournier (1999)
H_{Blood}	mmHg.mL/g	23125000	Fournier (1999)
H_{Mix}	mmHg.mL/g	23125000	Fournier (1999)
$Po_{2\,FF}$	mmHg	54.3	Shalgi, *et al.* (1972)
$Po_{2\,Blood}$ *(arterial)*	mmHg	95	Fournier (1999)
$Po_{2\,Blood}$ *(venous)*	mmHg	88	Fraser *et al.* (1973)
cHb_{Blood} *(arterial)*	mol/ml	9.9×10^{-3}	Anderson (2002)
cHb_{Blood} *(venous)*	mol/ml	7.4×10^{-3}	Anderson (2002)
Hct (arterial)	%	47	Anderson (2002)
Hct (venous)	%	37	Anderson (2002)
P_{50}	mmHg	26	Fournier (1999)
n	-	2.34	Fournier (1999)

The values used for the arterial solution provide a maximum limit to the effect and the values used for the venous blood provide a minimum limit to the effect. The real effect will fall between these two limits.

The predictions of the model are shown in Figure 5.3. It can be seen that very small amounts of blood contamination can have a very large effect.

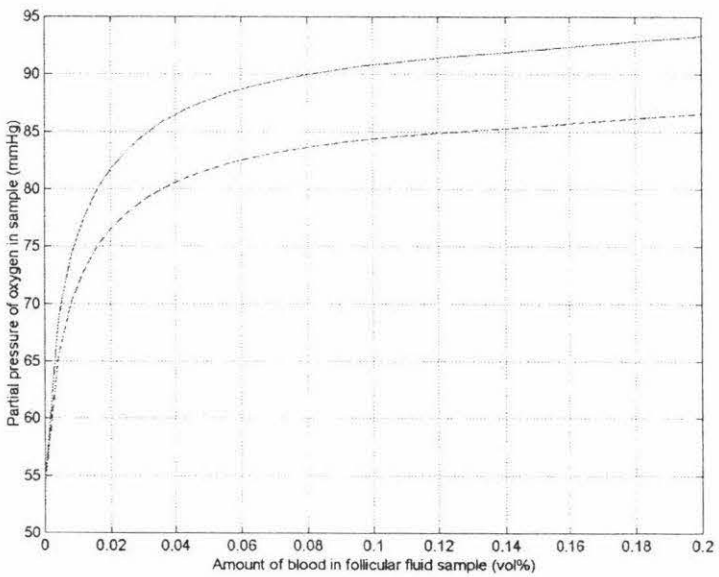


Figure 5.1 Predictions of the computer simulation of the release of oxygen from arterial blood (solid line) and venous blood (dash line) into a sample of follicular fluid

There is a requirement that the sampling device allows the user to gauge the extent of blood contamination in the follicular sample so that the sample can be tagged, since the presence of blood in the sample could assist the interpretation of the follicular dissolved oxygen data. An appropriate measure is the smallest amount of blood detectable by an experienced eye inside the sample container, which has been show by Levay, *et al.* (1997) to be $0.005^{vol}/_{vol}$ blood in follicular fluid. It is imperative that the sampling device allows detection of blood contamination to a proportion of less than 0.01vol%. This corresponds to a change in the oxygen partial pressure of the sample of approximately 20 mmHg.

5.1.2 Sampler materials

It has been shown that the material from which sampling apparatus is constructed can influence the quality of dissolved gas measurements. This occurs in syringes constructed from polypropylene and polystyrene (Scott *et al.*, 1971), and further, it has been shown that some polymers, such as polycarbonate and teflon having a greater effect than others, such as nylon, acetal and polyvinyl chloride (Stevens, 1992).

Clearly there is a requirement that a product that will be used for routinely sampling follicular fluid for dissolved oxygen measurement does not disrupt the dissolved gas equilibrium before the measurement is made.

Disposable syringes are made from common plastic materials. These materials often absorb an amount of oxygen from the environment, in the case of a syringe, usually the air. The amount of oxygen that is held within the plastic material is dependant on the polymer, the temperature and the partial pressure of oxygen in the environment. A step change in environment, say the syringe is filled with a relatively anoxic liquid, will result in a flux of oxygen from the syringe into the liquid, disrupting the initial liquid pO_2 until an equilibrium is reached.

5.1.2.1 System description

When the plastic of the syringe contacts a liquid with a lower oxygen partial pressure than the plastic, oxygen passes between the plastic and the liquid (follicular fluid), with a net flow from areas of high partial pressure to areas of low partial pressure. This flow is dependent on the geometry of the situation and transport properties of oxygen through the plastic and the liquid. However, the equilibrium partial pressure (after a long time) is independent of the transport properties and geometry.

The purpose of this model is to give an indication of the magnitude of the effect of different plastic materials commonly encountered in syringes on the partial pressure of oxygen in a sample of follicular fluid.

5.1.2.2 Formulation

An oxygen balance was applied over the solid (plastic) and liquid phases, taking into account the initial distribution and final distribution of oxygen between the two phases. The formulation makes a number of assumptions:

- Equilibrium is reached with respect to oxygen redistribution between the phases
- There is no exchange of heat or matter with the environment
- The system is isothermal and isobaric
- Follicular fluid obeys Henry's law of dissolved gases and the value of Henry's law constant is the same as that of blood plasma. This assumption is reasonable, given the similarity of composition (see section 2.1.4.2)
- A word equation for the oxygen balance:

O_2 in plastic initially + O_2 in FF initially = Final O_2 in plastic + Final O_2 in FF

$$V_p \times S_p \times pO_{2\,plas-initial} + V_{FF} \times \frac{pO_{2FF-initial}}{H_{FF}} = V_P \times S_P \times pO_{2.eq} + V_{FF} \frac{pO_{2.eq}}{H_{FF}}$$

Rearranging:

$$pO_{2.eq} = \frac{\left(V_p \times S_p \times pO_{2\,plas-initial}\right) + \left(V_{FF} \times \frac{pO_{2FF-initial}}{H_{FF}}\right)}{V_p \times S_p + \frac{V_{FF}}{H_{FF}}}$$

The solubility of oxygen in plastic materials can be found using the Arrehenius equation:

$$S_p = S_o . e^{\frac{E_s}{RT}}$$

With the following nomenclature:

Variable	Symbol	Unit
Volume of plastic material	V_p	cm^3
Solubility of oxygen in plastic at 37°C	S_p	cm^3(@STP)/cm^3.mmHg
Initial equilibrium partial pressure of the plastic	$pO_{2\,plas-initial}$	mmHg

Volume of follicular fluid	V_{FF}	cm^3
Initial equilibrium partial pressure of follicular fluid	$pO_{2\,FF-initial}$	mmHg
Henry's Law constant for follicular fluid	H_{FF}	$mmHg.cm^3/cm^3(@STP)$
Final equilibrium partial pressure of plastic and follicular fluid	$pO_{2\,eq}$	mmHg
Solubility pre-exponential factor	S_0	$cm^3(@STP)/cm^3.mmHg$
Solubilty activation energy	E_s	kJ/mol
Ideal gas constant	R	kJ/mol.K
System temperature	T	K

These equations were solved using a spreadsheet (MSExcel 97; Microsoft Corporation, Seattle, WA) for several different plastics and different mass ratios of plastic to liquid. The liquid was assumed to be at an initial pO_2 of 38 mmHg (5% of atmospheric pO_2). The results, shown in figure 5.1) show that there is a large impact on the equilibrium partial pressure of a liquid exposed to the material from which a plastic syringe may be constructed. It is worth noting also that the mass of the barrel of a 1ml syringe is typically 1-1.5g. The results from this calculation also show that there is a large difference between the impact of different plastic materials at low ratios of plastic to liquid. As the ratio increases the equilibrium partial pressure will tend toward the initial partial pressure of the plastic.

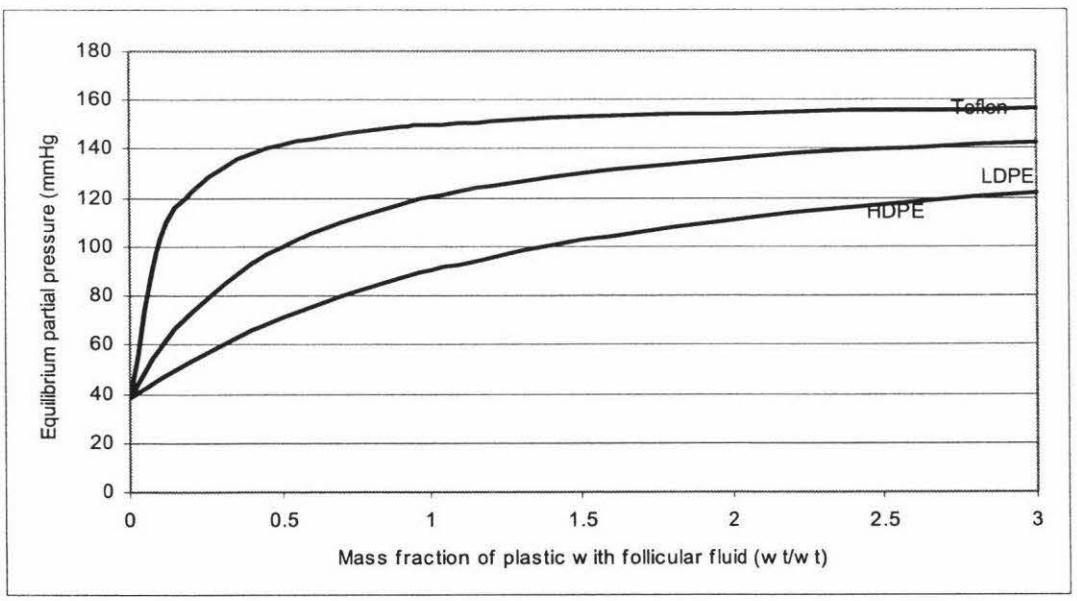


Figure 5.2 Calculated effects of different plastic materials on the final oxygen partial pressure of a sample of follicular fluid, initially at 38 mmHg (5% atm). The plastic materials were initially equilibrated with the atmosphere.

The question remains, then, how much change in the dissolved oxygen partial pressure is acceptable? The obvious answer is "Not so much that it makes a clinical difference". Unfortunately we do not yet know what change in follicular dissolved oxygen is significant in terms of predicting oocyte quality and so a limit of partial pressure

disruption can be set at ±20 mmHg (2.5 kPa), which is the value that is likely from the smallest detectable amount of blood contamination (section 5.1.1.2).

5.1.3 Bubble contamination

Bubbles of gas have been shown to have a disruptive effect on dissolved gas concentrations in systems where the liquid is not in equilibrium with the gas (Mueller *et al.*, 1976; Biswas *et al.*, 1982; C. G. Clark *et al.*, 1998; Müller-Plathe, 1998), which is a logical consequence of Henry's law of dissolved gases. It has also been shown that the rate of disruption is influenced by the extent of mixing between the liquid and gas phases (Ishikawa *et al.*, 1974). The rate, however, is not likely to be important, since the embryologist who is likely to make the measurement will not be available immediately. Therefore, we can assume that any bubble present in the system equilibrate the gas partial pressures with the liquid sample.

5.1.3.1 System description

The purpose of this model is to predict the effect on the partial pressure of oxygen in a sample of follicular fluid exposed to an air bubble before the measurement of dissolved oxygen.

Initially there is a liquid phase, whose oxygen partial pressure is lower than air and an air bubble. Oxygen will move between these two phases from areas of high partial pressure to areas of low partial pressure. After a period of time an equilibrium will be reached between the phases.

5.1.3.2 Formulation

An oxygen balance was applied over the system, taking into account the oxygen in the follicular fluid and in the air bubble. A number of assumptions were made:

- The system is isothermal
- There is no exchange of material with the environment
- The volumes of liquid and gas remain constant

- The system reaches equilibrium
- Follicular fluid obeys Henry's law of dissolved gases
- Henry's law constant for follicular fluid is the same as blood plasma

A word equation for an oxygen balance yields:

O_2 in FF initially + O_2 in bubble initially = Final O_2 in FF + final O_2 in bubble

More formally:

$$V_{FF} \times \frac{pO_{2FF-initial}}{H_{FF}} + \frac{pO_{2atm} \times V_{Bubble}}{R \times T} = V_{FF} \times \frac{pO_{2eq}}{H_{FF}} + \frac{pO_{2eq} \times V_{Bubble}}{R \times T}$$

Rearranging:

$$pO_{2eq} = \frac{V_{FF} \times \frac{pO_{2FF}}{H_{FF}} + \frac{pO_{2atm} \times V_{Bubble}}{R \times T}}{\frac{V_{FF}}{H_{FF}} + \frac{V_{Bubble}}{R \times T}}$$

With the following nomenclature:

Variable	Symbol	Unit
Volume of follicular fluid sample	V_{FF}	m^3
Volume of air bubble	V_{Bubble}	m^3
Henry's law constant for follicular fluid	H_{FF}	$mmHg.m^3/mol$
Initial partial pressure of oxygen in follicular fluid	pO_{2FF}	mmHg
Initial partial presure of oxygen in air bubble	pO_{2atm}	mmHg
Ideal gas constant	R	kJ/mol.K
Temperature	T	K
Equilibrium partial pressure	pO_{2eq}	mmHg

The equation for the equilibrium partial pressure was solved using MATLAB Version 5.3 release 11 (The Mathworks, Inc., MA, USA). The MATLAB m-file script is given in Appendix 2.

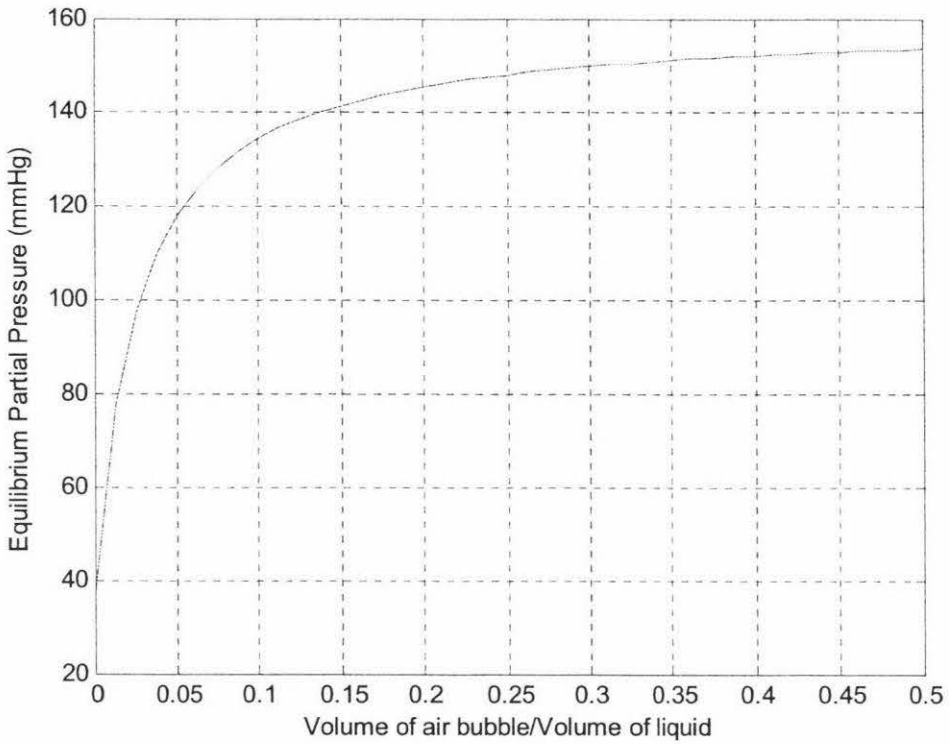


Figure 5.3 Calculated effect of an air bubble on the equilibrium oxygen partial pressure in a sample of follicular fluid initially at 38 mmHg (5%atm)

The predictions of the model are shown in figure 5.2. It can be seen that a relatively small bubble can have a large effect on the equilibrium partial pressure. This effect can be reduced by avoiding mixing in the liquid and gas (Ishikawa *et al.*, 1974).

This specification suffers the same weakness as that of section 5.1.2.2 in that we do not yet know how large a change in dissolved oxygen is clinically significant. Again it will be set at <20 mmHg (2.5kPa), and again, it is imperative that this condition be met.

5.2 Downstream compatibility

The purpose of collecting a sample is for the subsequent oxygen determination. It is, therefore, important that the sampler is capable of transferring the sample to the analytical apparatus without the sample dissolved oxygen being disrupted. This work does not consider the oxygen measuring equipment, but the design of the sampler should be to some extent driven by the nature of the analytical equipment. It is recommended that transferring the sample to the oxygen measuring equipment conform to the directions of the manufacturer of the oxygen analysing equipment.

5.3 Specification summary

The need statements from this chapter are summarised in Table 5.1. They pretain only to the module of the system that contains the follicular sample after it has been removed from the bulk fluid.

Table 5.1 Summary of needs statements relating to the physical aspects of sampling follicular fluid for dissolved oxygen determination.

Need Ref	Section Ref	Need statement	Rank*
5.1.1	5.1.1	Sampler allows detection of blood contamination	I
5.1.2	5.1.2	Sample container material should not affect the sample DO	I
5.1.3	5.1.3	Bubbles in the sample do not affect the measured DO	I
5.2	5.2	Transferring the sample to the measuring equipment conforms to manufacturer's instructions	R

The specification that are derived from the needs identified in this chapter are summarised in Table 5.2

Table 5.2 Summary of specifications relating to physical aspects of sampling follicular fluid for dissolved oxygen determination.

Spec Ref	Need Refs	Metric	Unit	Value
Sp20	5.1.1 5.2.1 5.1.3	Maximum change in a sample's dissolved oxygen partial pressure	mmHg	<20
Sp21	5.2	The sample container is compatible with the oxygen measuring equipment	Y/N	Y

5.4 Conclusion

The requirements for a device for sampling liquids for dissolved oxygen are clearly complex and multifaceted. A follicular sampling device must be designed to meet high scientific standards, as described in this chapter, while also meeting the specifications stipulated in chapters 3 and 4, so that it will be acceptable for clinical use.

The real test of these criteria, however, is the acceptance of such a sampling device in the fertility clinic.

Chapter 6. Design details of a working follicular sampling device and protocols for dissolved oxygen determination in a fertility clinic

> The message is that there are no "knowns". There are things we know that we know. There are known unknowns. That is to say there are things that we now know we don't know. But there are also unknown unknowns. There are things we don't know we don't know.
>
> US Secretary of Defence Rumfeld at NATO Headquarters, Brussels, Belgium June 6 2002

This chapter deals with the design of a follicular sampling device that complies with the specifications laid out in chapters 3, 4 and 5. The sampling device was to be designed so that it was acceptable to the clinicians involved in this study and would be used for preliminary measurements of dissolved follicular oxygen so that the oxygen concentration could be correlated with oocyte developmental competence.

The device is based on a prototype developed for measurement of follicular dissolved oxygen for research purposes (Redding, personal communication) and was not originally designed with the knowledge gained through time spent in the fertility clinics. This chapter describes how the device was improved in light of the consumer research described in the previous chapters.

6.1 Description of the sampling device

The device is a rectangular Perspex block, cut laterally into two slices. Each of the slices has an arc carved into the inside surfaces allowing a silicone tube to be placed through the block. The two slices of Perspex are attached to each other by pins, approximately 45mm long and are held together by two bolts. A circular channel approximately 9mm in diameter is carved into each of the slices which intersects the arc, allowing a syringe (with the same dimensions as a Hamilton 1ml gas tight syringe, Model 1001; Hamilton Company, Reno, NV, USA) to be inserted so that the needle pierces the silicone tubing and sits in the flow of follicular fluid.

The silicone tubing attaches to a nylon plug at each end and these in turn are inserted into smaller pieces of silicone tubing. A standard oocyte aspiration kit is severed in the middle of the PTFE tubing and these severed ends can be fitted inside the smaller sections of silicone at each end of the sampling device. The aspiration kit is fitted in

such a way that the flow of follicular fluid is in the opposite direction from fluid being drawn into the sampling needle.

When a sample is required, the operator inserts the syringe into the circular channel so that the needle pierces the silicone tubing and a sample of the flowing liquid can be drawn in the reverse direction from the main flow into the barrel of the syringe. The syringe is then drawn back out of the sampling device and the silicone tubing seals hermetically as the needle is removed.

The curve of the silicone is thought to reduce the chance of drawing an oocyte into the syringe. The curve of the silicone tubing also helps guide the sampling needle tip into the centre of the silicone tubing so the needle does not become embedded in the wall of the tubing, where there is no liquid to be sampled.

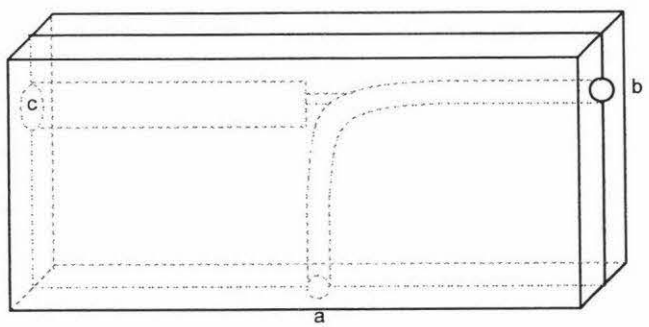


Figure 6.1 The sampling device. The whole device is a rectangular block that is split laterally into two parts. A length of silicone tubing lies in the curved, circular conduit and attaches to the tubing of the oocyte aspiration kit from where fluid enters (a) from the needle, through the silicone tubing and out (b) towards the collection tube. A port (c) allows a syringe to be inserted where the needle punctures the silicone tubing and a sample can be drawn out against the flow of follicular fluid.

The device uses disposable silicone parts so that the main pieces of the device that contact the follicular fluid and oocyte are not reused. The syringes, needles and perspex frame, however, are reused.

It is clear that this model does not satisfy all of the stipulations from chapters 3, 4 and 5. While that device had been tested to show that it did not damage oocytes or lose oocytes, it is not known whether the device has embryotoxic or teratogenic properties and the syringes are not amenable to easy sterilisation. The rest of this chapter describes the modifications that were made to the device and the protocols that were developed to allow its use in the clinical environment.

6.2 Minimisation of pre-analytical errors

This section describes the steps taken to reduce the preanalytical errors that would be introduced by such a system. In addition, the use of the Hamilton model 1001 syringe did not comply with a number of the stipulations previously described, most notably the ability to be sterilised (see section 6.8) and being compatible with a gas analyser (section 5.4).

The Hamilton syringe is composed of a polymer plunger, a glass barrel with a stainless steel collar at the needle end, a PTFE block that supports the needle at the end of the barrel, a stainless steel needle and another stainless steel collar that screws to the end of the barrel to secure the needle. This apparatus is amenable to autoclave sterilisation on the provision that it has been completely disassembled. It is highly impractical to disassemble, sterilise and reassemble such a syringe routinely while maintaining sterility and without damaging or losing the small parts.

Further, the syringe has a needle that is in essence fixed to the syringe while it contains a sample. Most clinical gas analysers require the sample to be injected from a luer fitting or to be aspirated through a capillary. The Hamilton syringe does not allow either of these options.

6.2.1 Evaluation of K-ATS-1000 syringe for sampling for dissolved oxygen

It was preferable to use a syringe that was already in common use in a fertility clinic, such as the K-ATS-1000 (Cook Australia, Queensland, Australia), since this syringe has a very small headspace (and thus a reduced chance of collection air bubbles – see section 5.1.2). However, this syringe is made from plastic and so may not be suitable. The K-ATS syringe is used during embryo replacement with a straw fitted at the luer fitting, containing the embryo(s). The syringe contains a small amount of liquid which flushes the embryo(s) from the straw. The syringe is designed to have a minimal headspace by using a plunger that is moulded with a prong that fits into the luer tip of the syringe barrel.

An experiment was conducted to assess the impact of the materials of construction of a type of plastic syringe that is accepted for use in IVF procedures (K-ATS-1000 syringe:

Cook Australia, Queensland, Australia) on the concentration of dissolved oxygen in a liquid sample.

6.2.1.1 Materials and methods

Human follicular fluid was obtained from combined fluid from hyperstimulated ovaries during conventional IVF, after the oocytes had been removed. 10 mL of this fluid was sparged with nitrogen gas (BOC New Zealand, Auckland, NZ) in a stirred, jacketed glass vessel and covered with a layer of Parafilm. The vessel containing the sparged follicular fluid was held at a constant temperature in a jacketed cell with a flow of water at 37°C. The apparatus is shown in Figure 6.2. As quickly as possible the 1 mL plastic syringes were filled with 700µl of the deoxygenated follicular fluid and the dissolved oxygen content was measured using a Diamond General oxygen electrode (Prod N° 733, Diamond General, Michigan, USA). The syringes were capped using polypropylene syringe caps (Prod N° 308341, BD, New Jersey, USA) and then incubated at 37°C and the dissolved oxygen was measured 20 minutes later using the same apparatus.

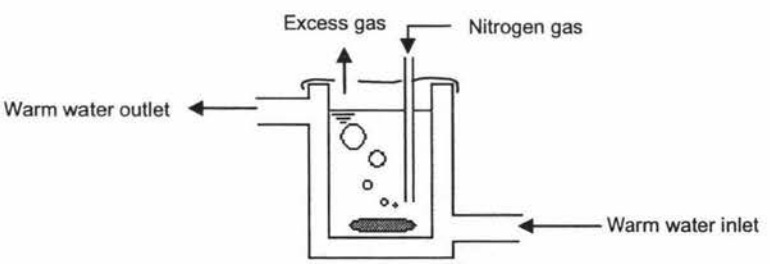


Figure 6.2 Diagram of apparatus used to maintain isothermal and anoxic conditions

6.2.1.2 Results and discussion

The average initial oxygen content was 8.2mmHg, while the oxygen content measured 20 minutes later was 119.3mmHg. An analysis of variance of the data showed that this difference was significant ($p<0.001$). Despite the attractiveness of using syringes that are routinely used in IVF procedures and the fact the syringes have no headspace (see Section 5.1.2) these syringes obviously alter the dissolved oxygen content of the follicular fluid to an unacceptable degree.

Previous studies have shown that mass transfer between glass containers and their liquid contents is not significantly large (Scott *et al.*, 1971; Restall *et al.*, 1975; d'Ortho *et al.*,

2001). The experiment was repeated using glass syringes. The average initial oxygen content was 28.1mmHg and the average dissolved oxygen content after 20 minutes was 32.3 mmHg. Analysis of variance in the data showed that this difference was not significant.

On this basis it was decided to use syringes with glass barrels that were compatible with the sampling device but complied more readily with the specifications than the original Hamilton syringe, especially in terms of ease of assembly and ease of sterilisation.

6.2.2 Effect of bubbles

Previous studies have prevented the formation of bubbles from the syringe's headspace by priming the syringe with a liquid heparin solution, which also prevents the sample from clotting (Fraser *et al.*, 1973; Fischer *et al.*, 1992), although the clotting potential of follicular fluid is low, given the exclusion of fibrinogen from the antral space.

The bubbles in a carefully collected liquid sample are often created from the gas that fills spaces in the syringe and needle before liquid is aspirated. These spaces illustrated diagrammatically and the effects photographically in Figure 6.3.

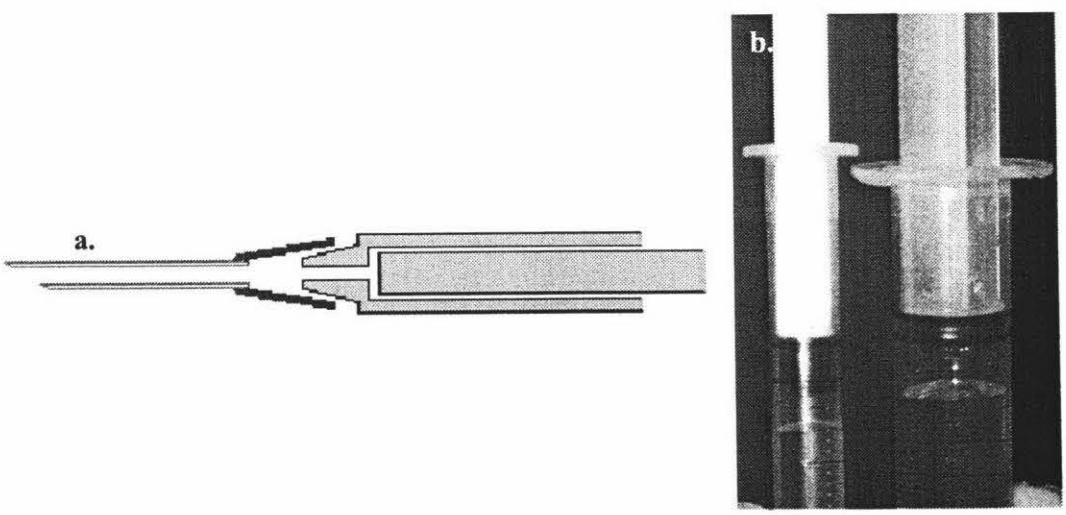


Figure 6.3 (a) Diagramatic representation of the end of a syringe with luer style needle attached. Note the space in the needle, top of the needle and end of the syringe. This becomes more apparent when the syringe is filled with liquid (b).

It was decided to prime the syringes with liquid (follicular flush media, at the clinician's request, rather than saline) prior to sampling, but also to exploit the phenomena previously described in which the bubbles have less effect on dissolved oxygen if the

syringe contents are not mixed (Ishikawa *et al.*, 1974). Thus, the samples were exposed to minimal movement and were held with the plunger end uppermost where possible.

A possible problem with adding an aqueous solution to a follicular sample is that the solution will contain its own dissolved oxygen. The calculation of the amount of oxygen in an aliquot of liquid is trivial.

6.2.3 Time delay before measurement

For the reasons described in section 5.1.3 it was decided that measurements would be made as soon after sampling as possible, meaning that the equipment required for measuring dissolved oxygen would be located at the fertility clinic. The analytical equipment would be located near to the site of aspiration to minimise the amount of handling of the specimens and reduce the likelihood of accidental spills and other undesirable events during transport.

6.2.4 Sample temperature control

Although it is stated in section 5.1.4 that the sample can be held at room temperature, this could violate the need to not damage the oocyte by exposure to temperature changes (section 4.1.2) in the unlikely event that the oocyte is accidentally aspirated with the follicular fluid into the syringe. The potential for a substantial temperature gradient and consequent damage to oocytes caught in the sample was reduced by preheating the syringes to 37°C and maintaining them at this temperature in the theatre using hot blocks during the oocyte recovery procedure. After the samples had been screened by an embryologist (see section 4.1.3) the samples were held at room temperature.

6.2.5 Blood contamination

A further advantage of using glass syringes, is that they can show the extent of blood contamination, as stipulated in section 5.1.3. To ensure that glass syringes allowed visualisation of the required levels of blood contamination a series of dilutions of whole blood were made in bovine follicular fluid. These solutions were aspirated into glass syringes. Inspection showed that the blood could easily be detected in the appropriate dilutions (figure 6.4).

In practice, blood contamination does not need to be quantified. Simply detecting the presence of any amount of blood indicates that a follicular sample is sufficiently contaminated to warrant its exclusion from dissolved oxygen determination.

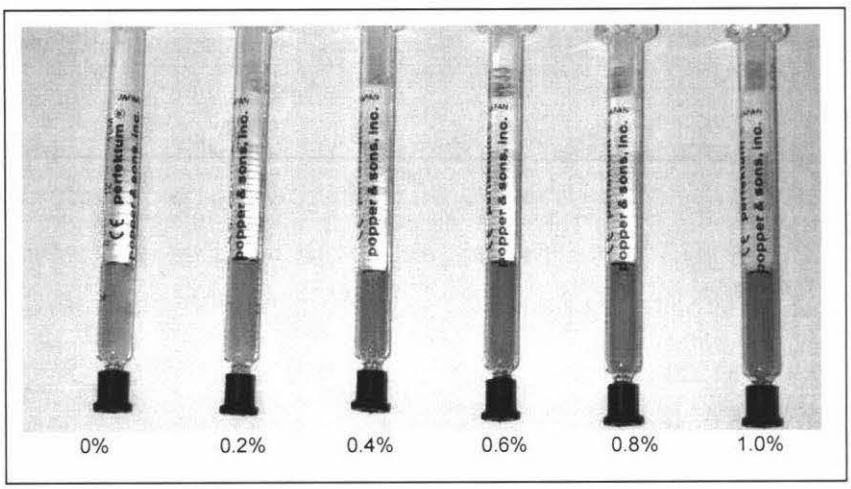


Figure 6.4 Dilutions of whole blood in bovine follicular fluid at a range of dilutions. Note that the extent of contamination is in volume percentages.

6.2.6 Fluid mixing within the needleset

It is required that the follicle from which a particular sample originated is recorded, as described in section 3.5.10.

An experiment was conducted to establish whether a sample could be drawn from the flow of follicular fluid immediately as the aspiration needle entered a new follicle. The purpose of this experiment was to ensure that a sample of liquid corresponded to a particular oocyte and follicular fluid and was not a sample of the previous follicle's fluid, left in the needle set, or a mixture of the two. It is in essence a tracer study performed over the oocyte aspiration kit, modified with the sampling device.

A tracer study is a technique where a trace is added to a fluid stream and the concentration of the tracer is measured after some predetermined time and/or distance of flow. The profile of the tracer concentration indicates the nature of the flow within the system and shows the extent of mixing or plug flow behaviour.

6.2.6.1 Materials and methods

The absorbance of an ink-water mixture was measured across the spectrum from 700nm to 350nm so that the optimal wavelength could be ascertained for the rest of the measurements.

Samples of frozen human follicular fluid (5-8 mL) were heated to 37°C and placed in a well mixed, jacketed vessel. The vessel was sealed. The seal had 3 ports through it, one to supply pressurised gas, one to inject a tracer and one to allow flow out, through the modified oocyte aspiration kit (see Figure 6.5). The system was pressurised to give a flow rate of approximately 5mL/minute (according to data collected at a fertility clinic, personal communication) and 0.1 mL tracer (blue ink) was injected when the fluid had filled the tubing of the oocyte aspiration kit. The outflow was collected into weighed plastic curvettes (Eppendorf Scientific, Inc., New York, USA) which were changed every 2 seconds. The collected fluid aliquots were weighed and diluted with 1mL of deionised water. The absorbance of the aliquots was measured at 600nm using a spectrophotometer (U2000; Hitachi High-Technologies Corporation, Japan). The extent of dilution was calculated for each aliquot and the original absorbance of each sample calculated.

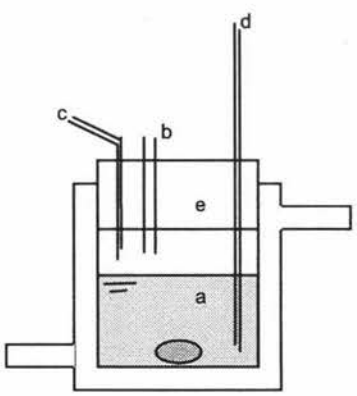


Figure 6.5 The apparatus used for the tracer study on the modified oocyte aspiration kit consisted of a jacketed cell containing follicular fluid (a) under pressure. The compressed air supply (b), the tracer inlet (c) the oocyte aspiration needle (d) transect a rubber bung (e).

6.2.6.2 Results and discussion

The ink-water mixture absorbance profile is shown in figure 6.6. It shows a peak at 600nm, thus determining the wavelength for measurements in the rest of the samples.

The absorbance at 600nm of each sample was plotted as a fraction of the difference between the original absorbance of the follicular fluid and the final absorbance. The results of three runs are shown in Figure 6.7. It can be seen that the flow can be described as generally plug flow with an initial delay. Thus the whole tube is filled with the liquid that results from a step change in the inlet composition of liquid after 1.5-2mL have passed through. Such a step change would be expected in the case of the needle entering a new follicle, and so it can be seen that a sample of liquid should be drawn after 1.5 – 2mL has passed through the set.

The noise in the data is likely to be due to effects of diluting small volumes of liquid and making measurements. The volume of each sample was generally between 0.1 and 0.2 mL and the volume of diluent was up to ten times the original volume. Thus small errors would have been multiplied by the dilution factor and become considerably larger. However, irrespective of the shortcomings in the tracer measurement, the result of the study is clear.

In practice this means that a sample should not be drawn until follicular fluid has been seen to be flowing into the collection tube, by which time 1-2mL will have accumulated in the tube and the system will have been purged of the previous follicle's fluid.

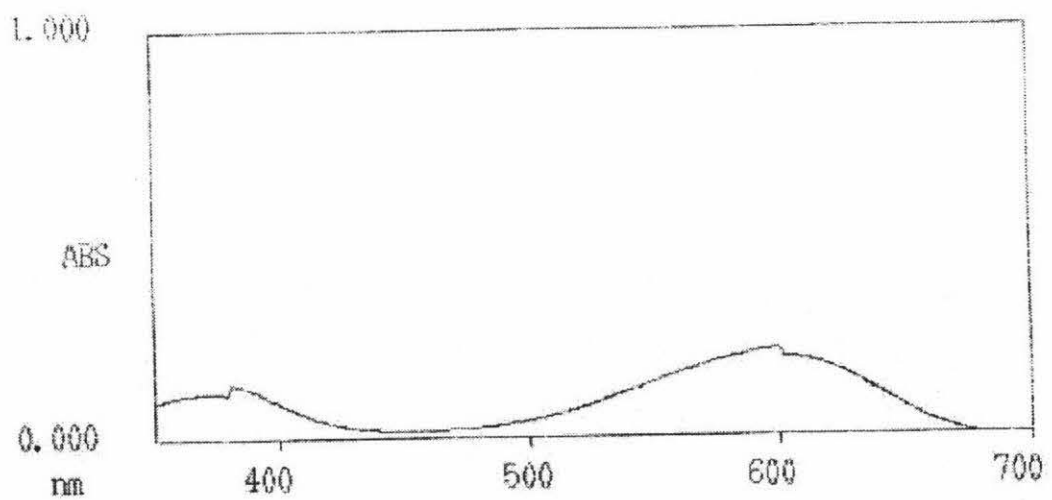


Figure 6.6 Absorbance of ink-water mixture from 700nm to 350nm. Note the peak at 600nm.

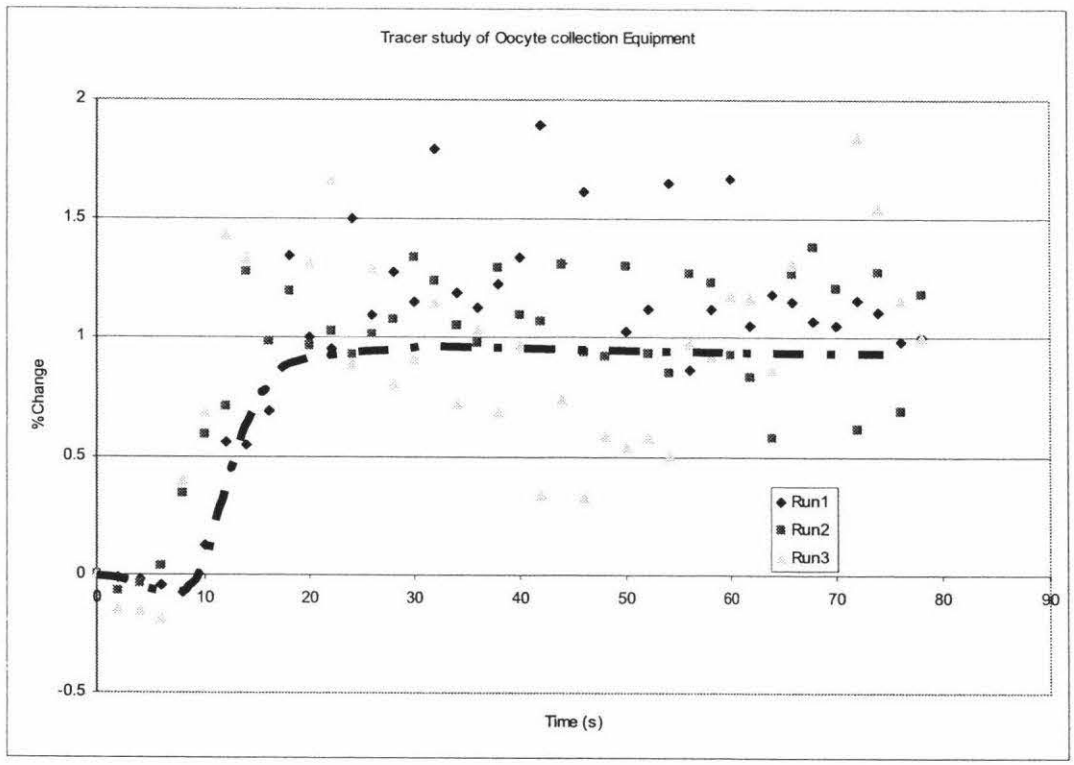


Figure 6.7 Absorbance of follicular samples with tracer added plotted as a proportion of the total change. Note the overlaid dot-dash line showing flow with little mixing but is delayed by 15-2.0mL.

6.3 Tracking samples and oocytes after sampling

A protocol was developed whereby the sample syringe would be held in pre-labelled tubes and the oocytes cultured separately in pre-labelled culture dishes. This would allow the sample and corresponding oxygen measurement to be linked to the oocyte from the same follicle.

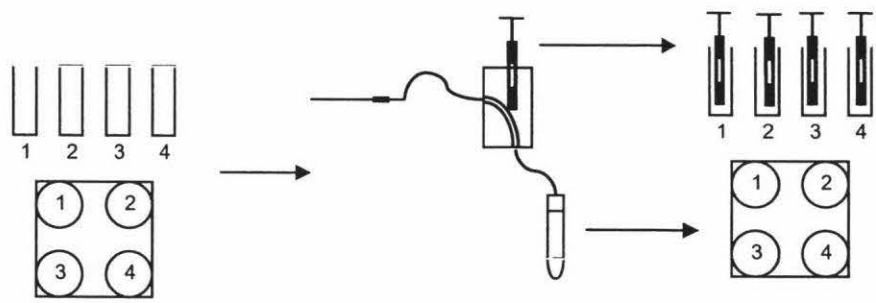


Figure 6.8 Samples and oocytes are tracked during the pick up procedure by labelling tubes and culture dishes are labelled before the aspiration. During the aspiration the sample syringes and oocytes are placed in the tubes and culture wells that corresponds with each other.

6.4 Minimise effect of shear forces on the oocyte-cumulus complex

As previously described in section 4.1.1, drawing an oocyte and its cumulus through a narrow bore needle could damage or worse, strip away the cumulus. In the most extreme cases, the zona pellucida could crack and the oocyte could completely lyse.

In order to prevent this, a wide bore needle was selected (18 gauge) but this caused punctures in the silicone tubing that would not self-seal. Originally the device was designed for a 21 gauge needle, which was narrow enough for the silicone to hermetically seal after the needle was withdrawn. The solution to this problem was to fit a needle into the device and to attach each syringe to the needle for sampling (figure 6.8). A consequence of this modification to the sampling method means that there could be periods where a portal exists between the interior of the aspiration kit and the environment. Through good communication the operator of the sampling device can ensure that a vacuum is not pulled while there is no syringe attached to the luer end of the sampling needle and this portal is open to the environment.

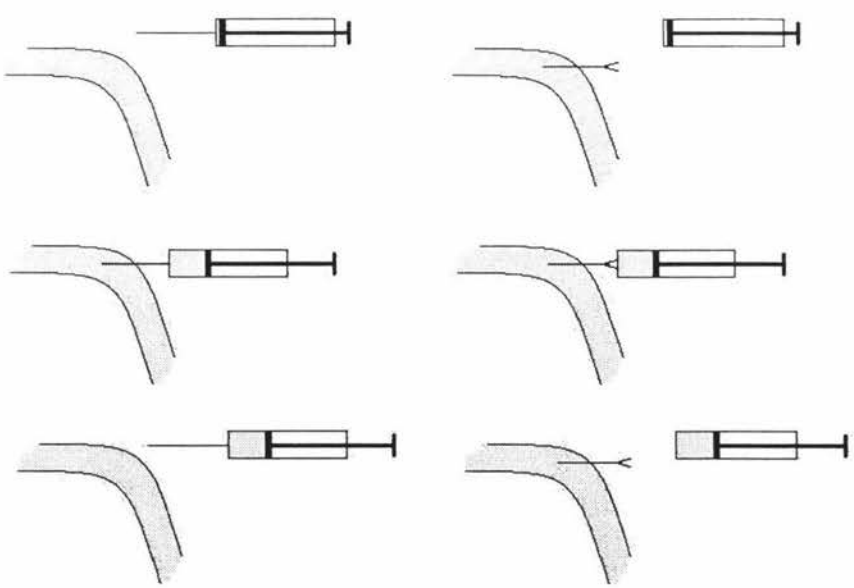


Figure 6.8 The original procedure is demonstrated on the left where a needle, attached to a syringe, is inserted into the silicone tubing, a sample drawn and the needle removed. On the right is the method modified to cope with the wider bore needle where the needle dwells in the silicone tubing and each syringe is attached and a sample drawn.

Previous work using this device had been carried out to ensure that there were no excessive shear forces acting on the oocytes as they passed through the silicone section of tubing and through the needle into the sampling syringe (in the event of an oocyte being drawn into the sample). Bovine oocytes were recovered from the ovaries of slaughtered cows and cultured until the cumulus had expanded. The oocytes (and cumuli) were drawn through the needle set in 5ml of bovine follicular fluid and a 1ml sample was drawn using a 21 gauge needle into a Hamilton syringe (Air tight syringe model 1001; Hamilton Company, Reno, NV, USA). 190 oocytes were aspirated and 21 (12%) were accidentally drawn into the sample. None of the oocytes suffered any damage to the cumulus mass or the zona pellucida that was detectable by light microscopy (Redding, personal communication).

In a second investigation, 19 pieces of blood fibrinogen clot were cut into pieces approximately 3mm in diameter and aspirated in human follicular fluid through the needle set with the silicone section in place and also drawn into the sampling syringe through an 18 gauge needle. The vacuum for aspiration was applied using a syringe, in the same manner as in a clinical setting. Each clot was photographed before and after aspiration and was judged not to have been damaged, taking into account the size of the clots (larger than a typical oocyte-cumulus complex) and the brittle nature of fibrinogen clots (the cumulus oophorus is more flexible than a fibrinogen clot). A typical example is shown in figure 6.9.

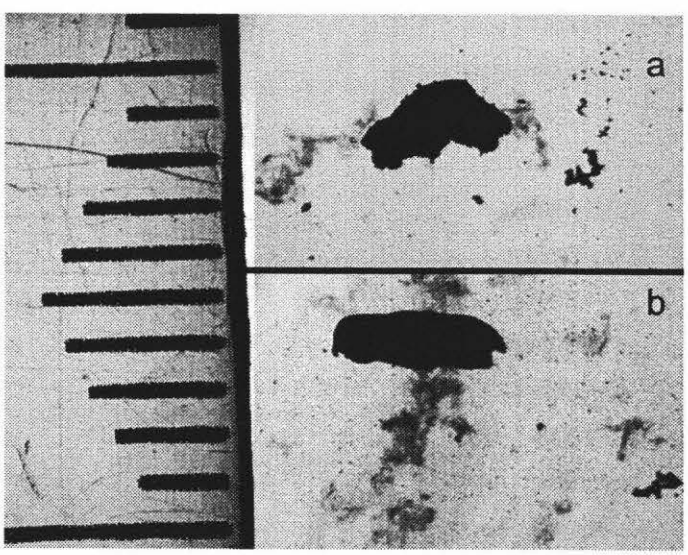


Figure 6.9 A piece of fibrinogen clot (a) before aspiration through the follicular sampling device and (b) afterwards. Notice the millimetre scale on the left panel.

In a third investigation (Coxhead, unpublished) the cumulus was mechanically removed from human oocytes and aspirated into the sampling syringe through an 18 gauge needle. No cumuli oophori were damaged, as judged by light microscopy.

6.5 Minimise temperature effects on OCC

The treatment of samples regarding temperature maintenance is discussed in section 4.1.2. The syringes were pre-heated to 37°C before the sample is taken and maintained at that temperature until examined for the presence of an oocyte to prevent the damage to the intracellular components.

6.6 Loss of oocyte

The stipulations from section 4.1.3 govern the whereabouts of the oocyte regarding the sample. Experiments of this nature have been described already in section 6.4. Through all of the experiments, no oocytes, fibrinogen clots or oocyte-free cumuli-oophori were lost from the system.

The proportion of the oocytes that were aspirated into the sample by Redding (personal communication) was higher than the specified limit. After a discussion, the clinicians were not concerned with this risk provided that each sample was screened for oocytes. Samples containing oocytes would not be amenable to dissolved oxygen determination as the oxygen balance would be disrupted during recovery of the oocytes from the sample.

6.7 Minimise embryo-toxic effects

It was vitally important to the clinicians involved in this study that the materials used in the sampling device were carefully selected and shown through independent testing not to be embryo-toxic. The criteria for this are given in section 4.2. For this reason significant effort was made during materials selection and testing in terms of embryo-toxicity.

6.7.1 Materials selection

The parts of the sampling device that would be in contact with the follicular fluid were made from materials that were known to be low-risk. This included the syringes, needles, silicone tubing and nylon connectors and syringe caps.

6.7.1.1 Syringes

The selection of syringes must be made according to several criteria, namely, not affecting dissolved gases in the liquid inside the syringe (see section 5.1.1), allowing visual inspection of the liquid contents for the presence of an oocyte (section 4.1.3), the ability to gauge the extent of blood contamination (section 5.1.3) and being suitable for sterilisation. The syringes available were constructed from glass (Popper & Sons, inc., NY, USA), glass, stainless steel and polytetrafluoroethane (Hamilton, Reno, NV, USA) or glass and stainless steel (Popper & Sons, inc.). None of the materials posed a threat of embryo-toxicity and were deemed suitable for sampling follicular fluid. The final selection of syringes was made based on the ease of sterilisation and preservation of dissolved gas composition. The embryo-toxicity of the materials of construction did not have a large impact on the choice made, since all of the syringes had glass barrels and were not intrinsically embryo-toxic and, since they were all made from glass, the need for visual inspection was satisfied in all three cases.

The final choice (figure 6.10) was made to use the all-glass luer-tip 1ml tuberculin syringes (Popper & Sons, inc., New York, USA) because these syringes were amenable to autoclave cycles, were constructed from materials that would not interfere with the oxygen balance, were not embryo-toxic, teratogenic or cytotoxic

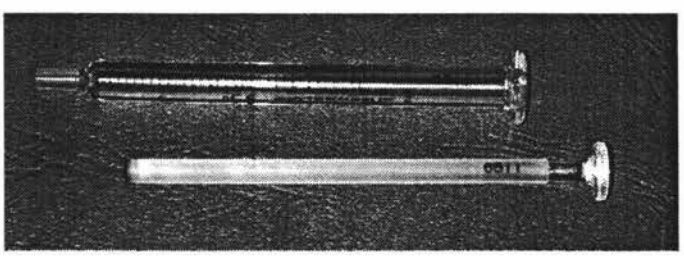


Figure 6.10 The syringe chosen for sampling follicular fluid was the glass luer-tip 1ml tuberculin syringe from Popper & Sons, Inc.

6.7.1.2 Silicone tubing

The silicone tubing was cured either with peroxide or platinum and came in several grades of smoothness on the interior surface. Platinum cured silicone (Cat# 95802-02 and Cat# 95802-05; Cole-Parmer Instrument Company, IL, USA) was selected over the peroxide silicone to prevent possible leaching of radical species, generated from the peroxide curing process, out of the silicone into the follicular fluid. The surface roughness of the silicone can cause proteins to adhere to the surface and have a detrimental impact on cleaning the interior of the tube. As the tubes were to be used one time only and did not require cleaning after the follicular fluid had passed through, it was thought that protein fouling and cleaning were not relevant issues.

6.7.1.3 Other materials

The other materials used were all in common medical use and consequently posed a very low risk of having embryo-toxic or teratogenic properties. These included the syringe caps (Cat # 308341, Beckton Dickenson and Company, New Jersey, USA) and sterile 1½" 18 gauge needles (Code NN*1838R, Terumo Corporation, Tokyo, Japan).

6.7.2 Materials preparation/cleaning

In addition to selecting materials that had a low risk of having a toxic effect on the oocytes, the equipment was washed according to the Fertility Associates protocol for materials that are likely to come into contact with oocytes or embryos. This protocol involves a four hour soak in 5% Pyroneg solution (Pyroneg, DiverseyLever New Zealand Ltd., Auckland, New Zealand) followed by six rinses in milliQ water. After rinsing, the items are dried in hot air and packed into Steriking (Wipak Medical, Wipak UK Ltd., Powys, UK) autoclave envelopes, sealed and steam sterilised. The glass syringes were wrapped in aluminium foil, packed into Steriking envelopes and autoclaved.

6.7.3 Embryo-toxicity testing with mouse embryos

The third stage of embryo-toxicity management was to have the materials tested according to the Monash Mouse Embryo protocol, which would further ensure that the materials were safe for use. The testing was carried out by Monash IVF (Queensland,

Australia) on the silicone tubing and nylon sections that were inserted inside the silicone tubing.

The testing involved mating BCBF1 female mice, 4-12 weeks old, and extracting the embryos from the oviducts, following cervical dislocation. The embryos were washed in Hepes-buffered Cook Australia culture medium and cultured overnight in Cook Australia Cleavage Medium in an atmosphere of 6% CO_2 and 5% O_2 in nitrogen. The next day, half of the 2-cell embryos were placed in dishes containing Cook Australia Blastocyst medium in which the silicone tubing and nylon junctions had soaked for 5 minutes. The other half of the 2-cell embryos were cultured in untreated Cook Australia Blastocyst medium as a control. The embryos were allowed to grow unchecked for 3 days. Afterwards, the media that had been exposed to the tubing showed 67% and 83% of the embryos had developed into blastocysts in dish one and 38% of the embryos had grown to blastocyst stage in dish two. These levels of blastocyst development were compared to the control wells in which 71% and 79% in dish one, and 41% and 47% had grown into blastocysts in dish two. The extent of blastocyst development in the test wells was deemed acceptable by the embryologist when compared with the control cultures, as the test wells showed a high proportion of embryos at the morula stage. The material was approved for human IVF use.

6.8 Sterilisation

The importance of sterilising equipment that is to come into contact with the patient, follicular fluid and oocytes cannot be overstated. It has been shown that pathogens, such as HIV and hepatitis C, can be transmitted by follicular fluid (Levy *et al.*, 2000; Passos *et al.*, 2002; Devaux *et al.*, 2003). Additionally, environmental organisms could contaminate and colonise the rich culture medium, having a serious impact on the oocyte/embryo and contaminating the incubation equipment. It is vitally important that the materials can be sterilised to reduce to risk the patient and embryo, but also to reduce anxiety among the clinical staff, who are charged with the well being of the patient and the efficacy of the treatment.

6.8.1 Material selection

The materials were selected with the amenability to sterilisation in mind, as well as the potential embryo-toxicity effects and other factors described. This was especially important in the selection of the silicone tubing and the syringes since these were the items that would contact the follicular fluid.

The main issue associated with sterilising glass syringes is thermal expansion of the constituent parts and the possibility of permanent deformation and fracture. Additionally, gas sterilisation requires that the gas penetrate all the spaces within a syringe, some of which are very small with minimal gas flow. Penetration is thus reliant on diffusion through the spaces, since glass is impermeable to ethylene oxide.

Hamilton syringes are made of many different parts, each of several different materials (Figure 6.11). The consequence of this fact is that each syringe must be disassembled, sterilised and reassembled aseptically. If a syringe were to be sterilised while assembled, it is likely that the metal parts would expand at a different rate from the glass and polymer parts, resulting in severe damage to the syringe. The manufacturer recommends that the syringes be disassembled before sterilisation.

A key problem is the aseptic reassembly of the syringe. It was likely that after repeated sterilisation cycles the parts would become damaged through excessive handling or lost altogether. Neither of these eventualities are attractive in a clinical setting.

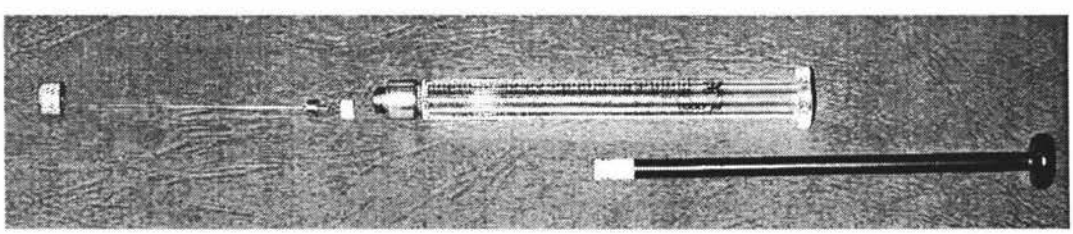


Figure 6.11 the Hamilton Air tight syringe, model 1001. Notice the number of parts, the stainless steel-glass syringe barrel and the small PTFE collar that ensures a good fit between the needle to the syringe.

All-glass syringes, such as the 1cc luer tip glass syringes (Cat #. 5202, Popper & Sons, inc., New York, USA) will withstand a heating-cooling cycle with little risk of damage, provided they are disassembled (ie. the plunger is not inside the barrel of the syringe). The reason for removing the plunger from the barrel of the syringe is to prevent fracturing the barrel as the parts expand and contract during the sterilisation cycle.

Syringes made from glass and metal (Popper & Sons, inc., New York, USA) are least suitable since they cannot be disassembled and the different expansion rates of glass and metal are very likely to break the glass parts of the syringe after repeated sterilisation cycles.

Clearly, of the syringes evaluated in this study, the 1ml glass tuberculin syringe with glass tip (item #5202, Popper & Sons, inc., New York, USA) was the most suitable and was selected for use in clinical trials.

6.8.2 Sterilisation regime

The choice of sterilisation method is limited to steam or gas sterilisation, due to the lack of gamma irradiation facilities in New Zealand. Steam sterilisation was chosen because a period of degassing is not necessary. This is particularly important in the silicone tubing, since the manufacturer states that the degassing requirements of the silicone tubing used are not well established, and it is possible that reactive by-products of the ethylene oxide treatment could leach out of the polymer matrix and into the stream of follicular fluid, containing the oocyte. Since the reactive species are not well-defined, the embryotoxicity potential cannot be accurately assessed and, as the adage goes: "It's better to be safe than sorry."

The syringes were dried after the wash protocol and wrapped, in a disassembled state, in aluminium foil and sealed in sterilisation envelopes (Wipak Medical, Wipak UK Ltd., Powys, UK), ready for an autoclave cycle.

The silicone tubing was dried after the wash protocol and packed, assembled, into autoclave envelopes for sterilisation. Furthermore, all packs were sealed with heat sensitive tape and packed into a paper bag for each autoclave batch, the paper bag also being sealed with heat sensitive tape. This ensured that it was plainly obvious if an item had been sterilised and the paper bag batches made it less likely that there would be subsequent contamination. The heat sensitive tape changes colour after it has been through an autoclave cycle, from white to white with black lettering with the statement "AUTOCLAVED".

The Perspex needle guide could not be sterilised easily, due to the fact that it could not withstand a large rise in temperature, which would be likely to cause a fracture due to

the metal parts and/or degradation of the polymer from the elevated temperature. This was thought not to be a considerable problem, since it was not to come in contact with the patient, follicular fluid or oocyte. After use the needle guide was washed thoroughly in de-ionised water, 70% ethanol solution and again in de-ionised water before being dried. This protocol was acceptable to the clinicians involved in this study.

6.9 Sampler retention volume

The hold-up volume in the sampling device was approximately 2.5ml. This was deemed unacceptable for the reasons given in section 3.5.5. The main concern was that the oocyte aspiration kit was now capable of containing an entire small follicle. This could cause delays in reporting whether an oocyte had been retrieved from a particular follicle, affecting the decision to flush the empty follicle with the hope of retrieving an oocyte.

It was decided that this volume problem could be solved by reducing the length of the silicone tubing. This was achieved by reducing the size of the Perspex block. Further advantages of this action are described in section 6.11.

The final volume of the tubing was approximately 1ml which significantly reduced the problems with liquid hold-up, disruption to the aspiration of oocytes from the patients' ovaries and errors in accurate sampling.

6.10 Sample volume

The volume required for measuring dissolved oxygen in a liquid sample is determined almost entirely by the analytical equipment to be used for the oxygen determination. The volume for a follicular sample was limited to being less than 600µl in section 4.5. The analytical equipment was selected and designed to use sample volumes as small as possible (see section 6.12.1 and section 6.12.2). The required volumes for the equipment described in these sections are less than 400µl, and so 400µl was set as the target sample volume, satisfying the specification stipulated in section 5.4.

6.11 Use of the sampling device

The way in which the sampling device was operated was improved by changing the syringe type. Originally a syringe with a fixed needle was used, presenting a substantial risk of needle-stick injury, given that a syringe containing a sample of follicular fluid or follicular fluid-blood mix was to be passed from the operator to an embryologist, examined and stored until the oxygen content could be measured, all the while having a needle attached (see figure 6.8).

The modified sampling device has a single needle that is attached to the syringe only when a sample is being drawn. After sampling the syringe contains the sample, the needle is left safely inside the perspex and the exposed end of the syringe (which is blunt) is covered with a polypropylene cap. This technique poses a very low risk of needle stick injury.

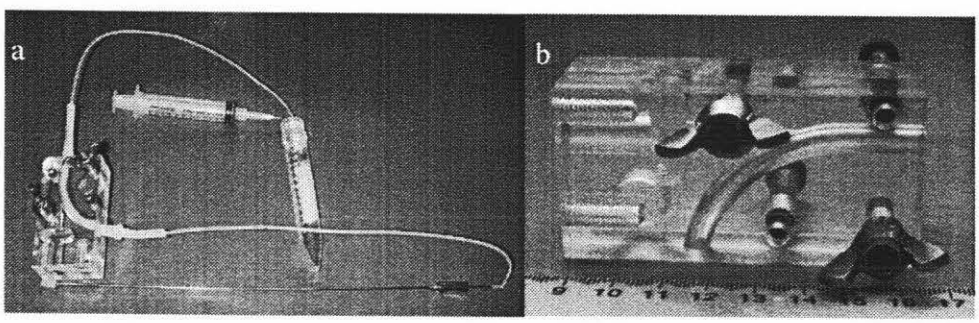


Figure 6.14 The original sampling device has been reduced and now holds a smaller volume, weighs less and is easier to hold. A whole modified oocyte aspiration kit (a) is shown and the sampling guide is shown with a scale (b).

6.12 Measurement of dissolved oxygen

Dissolved oxygen is most commonly measured using electrochemical means (the Clark electrode). This method provides a quick and reliable measure using technology that is well understood and has been in use for approximately 50 years (as discussed in section 2.6.3).

Two devices for measuring dissolved oxygen were investigated. One was a small Clark electrode (Cat # 733, Diamond General, Michigan, USA) and the other was a clinical device (i-STAT Corporation, New jersey, USA), which also used the principles described in the original electrode patent (L. C. Clark, 1959), in a small, self-contained device (see section 6.13.2).

6.12.1 Oxygen electrode method

This method was developed using an "off the shelf" oxygen electrode and a custom built housing to hold the sample in equilibrium until the measurement was made. As a consequence it was highly versatile and trouble shooting was easy, although with respect to its ease of use, it was a bulky piece of apparatus.

6.12.1.1 Oxygen electrode method description

The electrode (figure 6.14) consists of a platinum wire, housed in a glass rod. The glass rod and platinum wire are immersed in electrolyte solution (Cat#733A; Diamond General, Michigan, USA) and inserted into a plastic tube which screws into the threaded plastic body of the electrode at one end and at the other end has a PTFE oxygen-permeable membrane. When the electrode is polarised to –0.75V it produces a current that is proportional to the concentration of oxygen at the external membrane surface. This response is also affected by temperature and the composition of the electrolyte solution.

The electrolyte solution used was that supplied by the manufacturer of the electrode. This was used to reduce variability in the composition.

The measurements of dissolved oxygen were made inside a purpose built cell, designed to minimise temperature drift and exchange of oxygen between the sample and the environment.

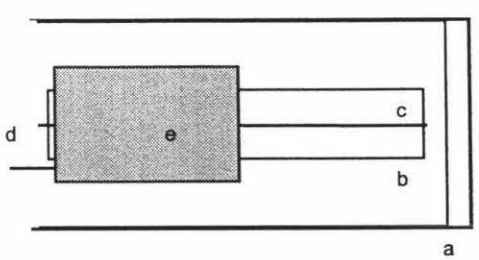


Figure 6.14 Diamond General mini Clark electrode. Oxygen diffuses through the PTFE membrane (a) and through the electrolyte solution (b) to the platinum electrode (c) that extends from the end of a glass fibre, where it is reduced, producing a current that can be measured across the contacts (d) between the platinum electrode and the silver reference electrode (e).

The cell consisted of a small transparent cylindrical dish with a plastic stopper, held in place by a rubber o-ring and sealed with silicone grease. This dish was placed inside a

transparent jacket, through which water was circulated from a temperature-regulated water bath. The cylindrical dish also contained a small magnetic stirring bar and the whole jacketed device sat atop a magnetic stirrer.

The cell is operated by injecting a bubble-free sample (approximately 300μl) through a hole in the stopper, withdrawing the injecting needle and replacing it with the oxygen electrode. The magnetic stirrer is activated and the measurement made from the response of the oxygen electrode. The sample is removed afterwards through the same route.

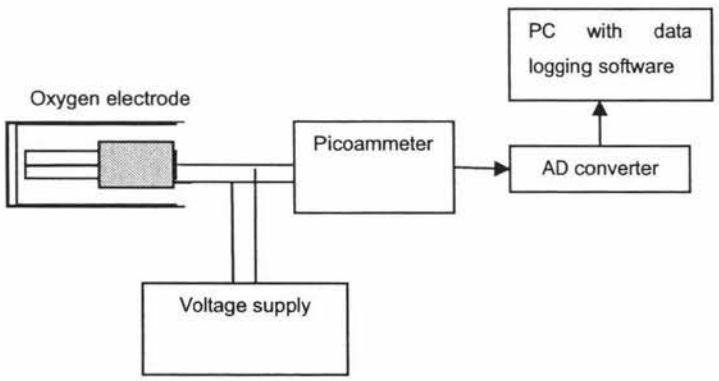


Figure 6.15 The oxygen electrode is polarised by a voltage supply and the current fro the electrode is converted by a picoammeter into a voltage. The voltage is digitised by an AD converter and the data is logged on a PC.

The electrode response was logged using a picoameter (Model 485; Keithley Instruments, Ohio, USA), which measured the current and produced a voltage signal proportional to that current. The voltage signal was measured and recorded using an analog-digital converter (Maclab 4e; ADInstruments PTY LTD, NSW, Australia) and the Chart recorder software (suppled with the Maclab 4e) on a desktop PC, as summarised in figure 6.15.

6.12.1.2 Oxygen cell construction materials

The materials from which the cell was constructed can have an impact on the quality of the measurements (Carignan & Gächter, 1994). In addition, it was required that the cell be transparent so that any gas bubbles could be easily detected and that the materials be amenable to being used for the construction of such a device (ie. that they are "workable"). It was decided that the cylindrical portion of the cell would be made from glass and that the cap, bearing a bore for injecting/aspirating the sample, would be constructed from acetal, with rubber o-rings to secure the two together.

Other options for the construction materials were to construct the cell and cap entirely from glass, which posed a problem with the workability and achieving a tight seal between the jacket and the cell, and the cap and the cell. There was also serious concern regarding the ruggedness of such a construction.

The purpose of the water jacket was to prevent temperature drift in the sample and oxygen electrode, since the response probe is highly sensitive to the operating temperature and must be calibrated at the temperature at which it is to be used.

6.12.3 Ensuring linearity of probe response

The manufacturer of the oxygen electrode recommends that the sample is mixed adequately to prevent formation of a oxygen concentration gradient between the outer membrane surface and the bulk sample. If the sample is not sufficiently mixed, the response of the probe can become non-linear with increasing oxygen concentration and the repeatability of measurements can be affected. In order to find an appropriate level of mixing, the probe was calibrated using three levels of oxygen concentration. The calibrant was tonometered bovine follicular fluid, obtained from ovaries from the local meat works the previous day. The follicular fluid was maintained at 37°C. The magnetic stirrer has a single control that is graduated arbitrarily from zero to ten, which does not indicate the absolute rate of stirring but does allow repeatable levels of stirring to be achieved.

The results are shown in figure 6.16 and they show that the response of the probe was linear at a setting of "3" but not at "1". Thus, any mixing above level "3" will be sufficient to disrupt any concentration gradient in oxygen concentration at the membrane surface. However, all subsequent calibrations were performed using three levels of oxygen concentration to ensure that the response was linear and that the amount of mixing supplied by the magnetic stirrer had not drifted.

It was considered prudent to use human follicular fluid for any subsequent calibrations, instead of water, which, while being a safer option clinically, introduces rheological differences and other potential matrix effects, which could interfere with the transfer of oxygen from the liquid to the membrane surface.

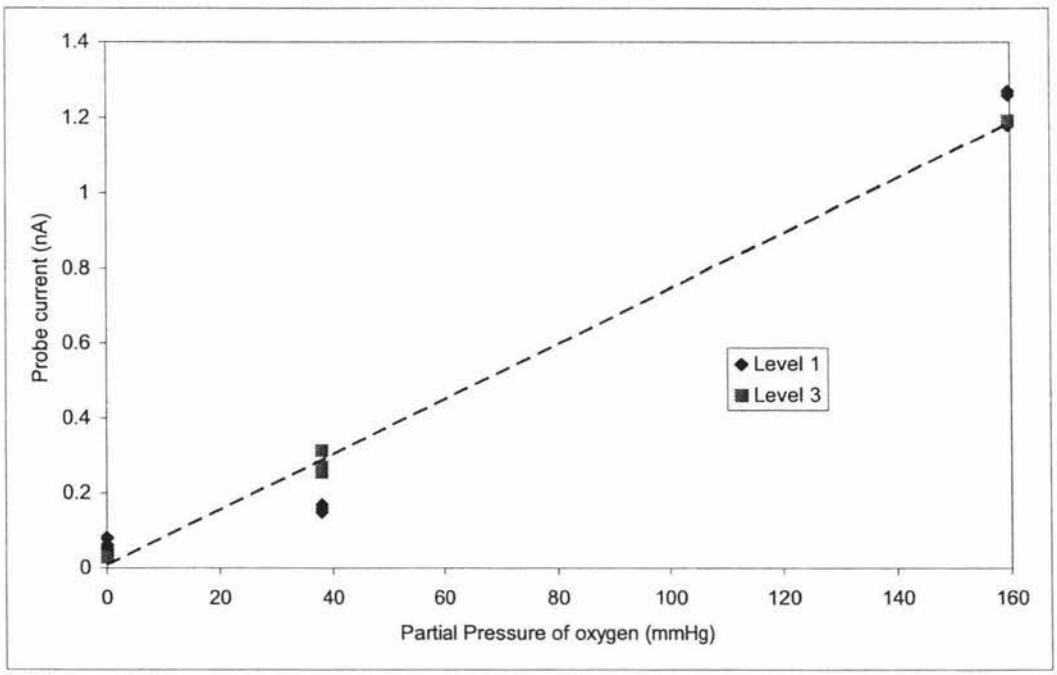


Figure 6.16 The linearity of the oxygen electrode is dependent on the extent of mixing. The mixing level is in the arbitrary units marked on the magnetic stirrer. Clearly "level 3" is sufficient to give a linear response, where "level 1" is not.

6.12.2 iStat method

The iStat (i-STAT Corporation, New jersey, USA) is a point of care clinical analyser, capable of measuring a range of physiological parameters of interest to the clinician. It is a highly engineered system, which is easy to use and reliable but operates largely as a "black box".

6.12.2.1 Description of the iStat analyser

The iStat system consists of two parts. The first part is a handheld device, which gives the results on a liquid crystal screen and is capable of transmitting the results to peripheral printing and data management devices (figure 6.17). The hand-held part also contains the calibration information for the various batches of test cartridges. The power is supplied from a pair of 9V batteries, housed in the back of the device. The front of the device has a numerical key panel so that patient information can be entered and results can be retrieved.

The second part of the iStat system is the test cartridge (figure 6.18). The cartridge contains a series of channels, through which the sample and calibrant flow, a sealed pouch of calibrant and a number of analytical surfaces connected to electrical contacts that interface with the hand-held part of the iStat. The test cartridges are made from

plastic and are designed for single use. There are several different types of cartridge, each designed to measure a different set of physiological parameters (eg. E3+ measures potassium, sodium, haematocrit and calculates haemogobin; CG8+ measures pH, pCO_2, pO_2, sodium, potassium, ionised calcium, haematocrit, glucose and calculates $[HCO_3^-]$, total CO_2, base excess, haemoglobin saturation, and haemoglobin concentration). The cartridges selected for this study were the G3+ (i-STAT Corporation, New jersey, USA) type, which measures pO_2, pCO_2, pH, and calculates $[HCO_3^-]$, total carbon dioxide, base excess, and haemoglobin saturation. The cartridges must be stored at 4°C.

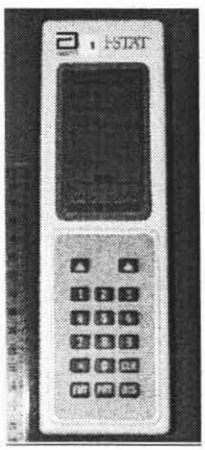


Figure 6.17 The iStat handheld analyser collects the data from the test cartridges, displays the test results and stores the test results. The test cartridges are inserted into a slot at the bottom of the instrument.

The sample volume is approximately 100μl. The time required for a test to be completed is 120-180s.

The iStat was designed for measuring clinical parameters in human blood, and not in human follicular fluid. The iStat was also designed for measuring gas concentrations that would typically be found in human blood and not the levels that one would expect to find in human ovarian follicles, which, according to the model and predictions of Gosden, *et al.* (1986) could be relatively anoxic. Thus, the intended use of the device for this study takes it beyond its design specification and so the capability of the iStat was validated for the purposes of this study.

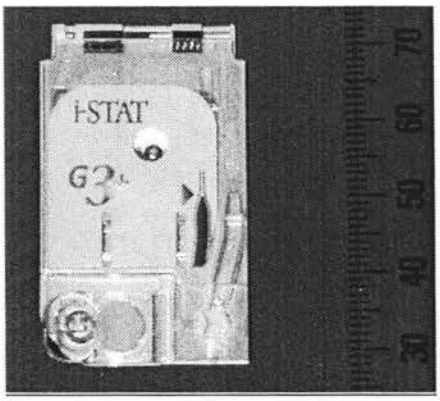
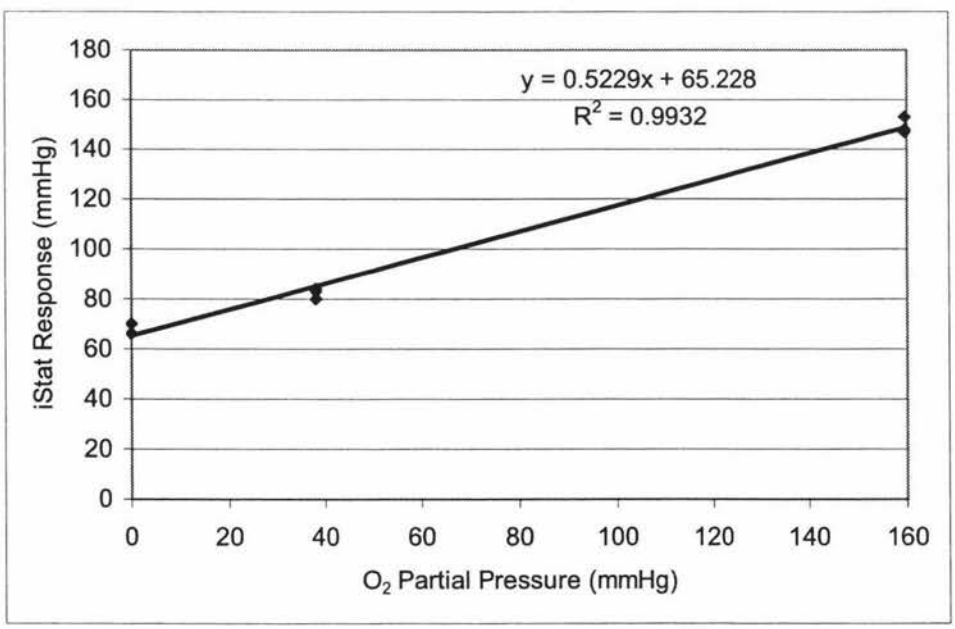


Figure 6.18 The iStat G3+ cartridge. The sample is injected into the port at the lower right. The Metal bands at the top are the sensors and electrical contacts for the hand-held part and the calibrant is stored in a metal pouch under the white sticker.

6.12.2.2 Validation of iStat pO_2 measurements with non-blood liquids

The iStat G3+ cartridges were warmed to room temperature, according to the manufacturer's recommendation. Human follicular fluid was tonometered at 37°C with gases with three different levels of oxygen concentration, namely, 0% O_2 (nitrogen), 5% O_2 in nitrogen (BOC New Zealand, Auckland, New Zealand) and 21% O_2 in nitrogen. An aliquot of liquid was drawn into a glass syringe (Cat #. 5202, Popper & Sons, inc., New York, USA) and injected into the G3+ cartridge. The cartridge was immediately inserted into the iStat device and the oxygen level was recorded. This was performed using fresh cartridges for three replicates at each level of oxygen concentration.

Figure 6.19 The iStat can be calibrated against human follicular fluid equilibrated with gases of known composition.



There is a discrepancy between the manufacturer's specification of the gas and the measurement of the iStat. However, the iStat can be "calibrated" against human follicular fluid equilibrated with gasses of known composition. This curve is shown in figure 6.19. It can be seen that the response is quite linear.

It is likely that the discrepancy is caused by oxygen leaching out of the plastic from which the cartridge is constructed. This effect would be less obvious in the presence of haemoglobin, since the haemoglobin can buffer changes in the dissolved oxygen partial pressure in whole blood. Furthermore, this effect will be more marked as the dissolved oxygen equilibrium of test liquid moves away from atmospheric (ambient) partial pressure.

6.13 Conclusion

It has been demonstrated in this section that a follicular sampling device can be designed that largely complies with the specifications stipulated in chapters 3 and 4. Furthermore, such a sampling device was acceptable to the clinicians for use in a fertility clinic.

While an acceptable follicular sampling device can be designed and built, the performance in the clinic has yet to be proven. An issue here is that there is no benchmark for performance of follicular sampling devices, since at present there is no such product.

Chapter 7. Performance of the sampling device in the clinical environment

Success is the ability to go from one failure to another with no loss of enthusiasm.

Sir Winston Churchill (1874 - 1965)

This chapter discusses the performance of the sampling device and protocols described in chapter 5 in the fertility clinic. Patients were recruited in two clinics, one in Hamilton and one in Auckland, New Zealand over the period August-November 2004.

Patients were informed about the study and asked if they wanted to participate. The patients were approached by the counsellor at the Hamilton clinic as they explained the IVF process and then again by the clinician when the patients had the ovarian ultrasound scan prior to induction of ovulation. Patients at the Auckland clinic did not routinely have a session with the counsellor and were approached by the clinician at the time of induction of ovulation.

Patients were then assessed by the clinicians for adequate ovarian response to hyper-stimulation and for other factors (such as ovarian adhesions) that could make the trial of the sampling device difficult or vice versa.

In total four patients gave their consent and were certified to proceed with the study by their clinician. Two patients were in Hamilton and two were in Auckland. There were several reasons for such a low volume of participants. Frequently the patients objected strongly about any deviation from "normal" IVF treatment. This is understandable since the treatment is inherently invasive, both physically and emotionally, and most of the patients did not feel comfortable being involved in a study that could impair their chances of pregnancy, regardless of the care that had been taken to prevent adverse effects to the efficacy of the treatment.

Several patients gave their consent to the study but the clinicians did not feel that was appropriate for physiological reasons.

Confounding these reasons was also a lack of enthusiasm from the clinicians after some initial problems (detailed through this chapter) with the sampling equipment.

7.1 Ethical considerations

The study was approved by the Waikato Ethics Committee (Hamiton, NZ) to sample follicular fluid during the course of routine IVF egg collection in order to measure dissolved oxygen, and potentially other substances, without inducing artefacts due to the sampling technique.

Excluded from the study were women who had 5 or fewer follicles on the last ultrasound examination before egg collection so that in the event of damage occurring to an egg during the sampling, the efficacy of the treatment would not suffer excessively.

The Waikato Ethics Committee reference number for this approval is WAI/04/02/007.

7.2 Clinical protocol

Once the patient had given their informed consent and the final human chorionic gonadotrophin (hCG) injection had been administered to induce ovulation, the author was contacted by the charge embryologist. This allowed approximately 33 hours between notification of an oocyte pick up and the procedure. This period was used to ensure that the sampling device, silicone tubing and syringes were cleaned according to the protocol and sterilised.

Immediately before the pickup procedure, the investigator assembled the sampling device and the standard aspirating kit inside a linear flow hood, observing aseptic technique on sterile surfaces. It is worth noting the difficulty involved in integrating a sterile component (ie. the aspirating kit and silicone tubing) with a non-sterile component (ie. the perspex sampling device), since the operator was only able to touch the sterile equipment **OR** the non-sterile equipment, and never both.

The sterile parts of the sampling device were held inside a sterile wrap and placed on the bottom of an equipment trolley in the theatre. The investigator, wearing sterile gloves and a sterile apron or scrubs was positioned at the patient's waist, facing the surgeon and nurse.

The theatre was populated by a number of clinical and non-clinical personnel. There were two nurses; the "drug nurse", who was responsible for the comfort and safety of

the patient, the "scrub nurse", who assisted the surgeon. The drug nurse was positioned at the patient's right hand side while the scrub nurse sat inside the patient's left knee. The patient's partner sat at the left side of the patient's head. The surgeon was positioned inside the patient's right knee and the embryologist was at the patient's feet with a trolley containing a microscope, heated media for oocyte washing and culture dishes.

A second embryologist was in attendance to exchange sample-containing syringes with fresh ones, to cap the sample-filled syringes and to examine them for the presence of oocytes under a dissecting microscope.

The first embryologist, in addition to keeping track of the number of follicles aspirated and number of oocytes retrieved retained the individual follicular fluids, after the oocytes had been retrieved and washed. This fluid was centrifuged, chilled and packed for further analysis.

After the surgeon had visualised the ovaries using an ultrasonic probe, aspiration would commence, with an unmodified aspiration kit. Suction was applied to the aspiration kit by the scrub nurse, using a syringe plugged into the top of a polystyrene collection tube. In all cases, in the interests of the patient's comfort, all of the follicles of the first ovary were drained using the unmodified kit (even though only 6 follicles were specified by the ethics committee). The modified kit was employed for aspiration of the second ovary.

When the surgeon punctured a follicle, and as the nurse applied vacuum, the operator of the sampling device withdrew the plunger on the sampling syringe. The target sample volume was 400μl. Effective communication regarding the application of vacuum and insertion of the needle through the follicle walls was vital, since the sampling syringe had to be in place for any suction to be applied. If the sampling syringe was not in place, the interior of the aspiration kit was open, via the silicone tubing and sampling needle, to the environment and air would leak in through the needle port.

It was decided that in the event of a low-grade failure, aspiration would continue using the modified kit but no follicular fluid samples would be taken into the sampling syringes. As a precaution, a spare, un-modified aspiration kit was kept on-hand in case of a catastrophic failure of the modified aspirating kit. If deemed necessary, the needle

of the modified kit would be withdrawn from the ovary and replaced with the un-modified needle, although this is undesirable because of the considerable discomfort to which the patient is subjected during this changing-of-the-needle.

7.3 Performance results

It was decided between the clinic's science directors and the investigator that the first few sampling opportunities would be used to find and resolve any unforeseen teething problems that arose during the sampling process and that the samples would not be used for oxygen analysis.

The aseptic assembly of the sampling device was difficult, particularly threading the sterile silicone tubing into the non-sterile perspex needle guide, without contaminating the ends of the silicone tubing, which were connected to the sterile aspiration kit. The modified needle set was used during aspiration of the second ovary and three follicular samples were obtained. The samples were drawn by an embryologist and were examined immediately under a microscope for the presence of an oocyte and none were found. At the time the equipment for measuring dissolved oxygen was not available and so the focus was on the sampling technique in this case. The embryologist that drew the samples expressed later that she had had a little difficulty drawing the syringe plunger and recovering the samples and that the area around the patient's legs was extremely crowded (with the clinician, the nurse and the embryologist). However, there were no problems experienced regarding communication between the clinician, nurse (who was applying suction) and the embryologist who was taking the samples.

On the second opportunity for sampling difficulty was experienced again during the assembly of the sampling port and the aspiration kit. The patient had their first ovary aspirated using the un-modified aspiration kit. The modified aspiration kit was used for oocyte collection in the second ovary. As suction was applied air began to leak into the kit through the hole in the silicone tubing where the sampling needle had been inserted. Collecting samples in the presence of air bubbles proved to be extremely difficult since the bubbles were introduced up-stream of the tip of the sampling needle and copious quantities of air were included in the samples. As the follicular aspiration continued the air leak worsened until it was decided to abandon the modified kit in favour of the original aspiration needle.

The third volunteer for the study was treated in the same manner, with the first ovary being aspirated with an un-modified kit and the second using the modified needle. The third attempt to collect samples was virtually identical to the second, with large amounts of air being drawn into the liquid stream through the hole in the silicone tubing. On this occasion the effect was more pronounced, to the extent that the suction was barely sufficient to draw the follicular fluid from the ovary, such was the volume of air introduced into the system. There was clearly a design flaw in the sampling device that needed immediate attention, so that the clinicians' confidence in the project did not deteriorate any further.

7.4 Iterative improvement

A second design was developed with consultation between an engineer, a biotechnologist, a product developer and the engineer who would construct the device (figure 7.1). This improved sampling device addressed the problems of gas leaking and had improved aseptic performance, since it had proven difficult to assemble the sterile silicone tubing with the sterile aspiration needle and the Perspex block that had not been sterilised.

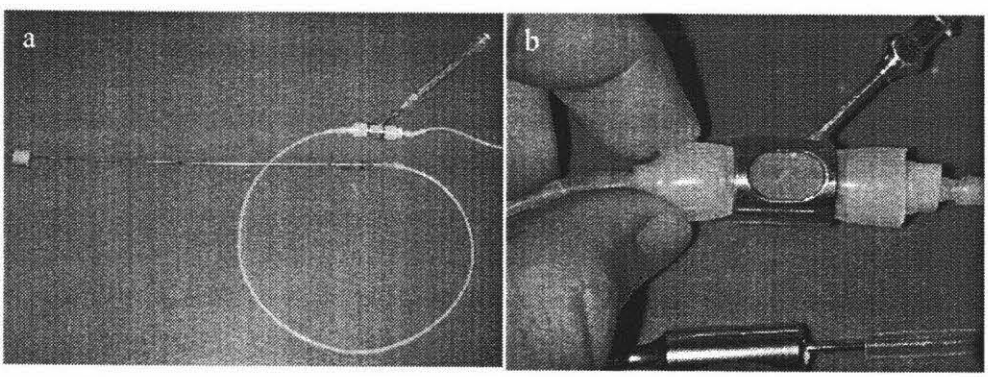


Figure 7.1 The improved sampling device. (a) the device in-line and (b) in detail.

7.4.1 Improvements on airtightness

The source of the air that leaked into the stream of follicular fluid was the interface between the needle and the silicone tubing. The silicone tubing did not form a tight seal after the needle was inserted and when a vacuum was applied inside the aspiration kit, air leaked past through the silicone. The strength of the seal was increased by replacing the silicone tubing with a section of tubing with a thicker wall (Cat# 96400-15; Cole-Parmer Instrument Company, IL, USA).

Since the new tubing was made from the same material as the old tubing, embryo-toxicity was not an issue. The thickness of the wall of the new type of tubing was 3mm.

7.4.2 Improved aseptic performance

The tubing was held using a stainless steel sleeve that had a guide mounted to the side to allow the insertion of the 18 gauge sampling needle. The metal sleeve also had a window cut into the side so that the flow of follicular fluid could be easily seen through the silicone tubing.

This system (the silicone tube and steel sleeve) could both be autoclaved, after being washed and pre-assembled, allowing improved aseptic performance.

7.4.3 Improvements regarding ergonomic factors

The new sampling device was considerably smaller, lighter and less "boxy" than the previous sampling device, consequently it was much better suited to use in the cramped surroundings of the oocyte pick-up procedure.

The sampling device was now small and light enough that its weight could be supported by the PTFE tube that links the aspirating needle with the collection tube.

7.5 Performance of the improved sampling device

The performance of the improved sampling device was trailed in the laboratory and then had a single use in the clinic. The laboratory trials showed that the device functioned well for sampling streams of liquid aspirated from a beaker under vacuum up to -70kPa. At higher vacuums the silicone began to leak air bubbles into the system. It was deemed acceptable that the device could withstand this level of vacuum.

This performance was shown to be repeatable after the device had been assembled and steam sterilised, showing that treatment with steam had no detrimental effect.

The trial in the fertility clinic showed that the device was much easier to handle when it was completely sterile, with none of the difficulties experienced in the past with the perspex that had not been sterilised. This substantial improvement in handling suggests that a specification should prescribe that a fully sterilisable device is imperative.

The improved sampling device was found to be much easier to handle and operate than the previous iteration as its size and weight had been reduced. However, while the extent of air leakage into the system had been reduced, it had not been cured completely. It had been reduced enough that the modified aspiration kit could be used to complete the oocyte pick-up but that the samples were still affected by extensive bubble contamination. As discussed earlier, the presence of bubbles can have a very large effect on the equilibrium partial pressure of gas in a liquid sample, and so the presence of bubble contamination in the samples was undesirable.

It was noted during the sampling that the plunger on the syringe was difficult to withdraw. This was due to the high vacuum inside the aspiration kit, resulting in a kind of "tug-of-war" for the liquid between the vacuum being applied by the nurse and the vacuum resulting from the operator of the sampling device attempting to withdraw the plunger of their syringe. Not only was it difficult to draw the liquid into the syringe but it was also difficult to retain it inside the syringe since the slightest relaxation would suck the plunger of the sampling syringe down, injecting the follicular fluid back into the flow from the ovary to the collection tube.

7.6 What went wrong?

Clearly the problem lay in an inadequate model of ovarian aspiration in the laboratory trials. The ovarian follicle, imbedded in a woman's abdomen, does not behave like a beaker of liquid, aspirated by the use of vacuum pump.

Firstly, the resistance to flow from the follicle increases as the liquid is removed and eventually the follicle has completely collapsed about the end of the needle (at which point the resistance to flow is very high).

Secondly, the vacuum supplied by the scrub nurse was not constant. It was supplied by withdrawing the plunger on a 25mL syringe. The strength of the resulting vacuum is then determined by the gas volume in the collection tube (and the rest of the kit) and the distance that the plunger is withdrawn. Two solutions were devised for this problem. The first was to limit the vacuum applied by the nurse through limiting the size of the syringe. Rather than using a 25ml syringe, a gentle vacuum could be applied by using a 5- or 10ml syringe. Laboratory testing showed that in addition to the sampling device

not leaking air bubbles, the sample could easily be drawn into the sampling syringe against the aspiration vacuum.

An alternative, was to use a commercial IVF vacuum pump. These often come with a pressure control mechanism, although the manufacturers suggest mild pressures be used, in the region of –80 to –100 mmHg (-11 to –13 kPa). This level of vacuum can be compared with the effect of using a syringe which can give much higher vacuum pressures when suction is applied. While it can be higher, it is also extremely variable (a simple calculation using Boyle's law shows that a 10ml collection tube half filled with liquid and at atmospheric pressure will, when a syringe plunger is pulled to 10ml, gives a vacuum of approximately –503 mmHg or -67kPa). Thus it can be seen that it is imperative that a sampling device be capable of withstanding relatively high vacuums or that the vacuum pressure is maintained at a sensible level.

It is likely that lowering the vacuum pressure during aspiration would reduce the flow rate of liquid from the follicles and thus the length of time required for the oocyte pick up. However, there are also subtle effects on the oocytes. It has been shown in animals that increasing the vacuum pressure increases the proportion of follicles that yield oocytes, but that increasing the vacuum also reduces the ability of those oocytes to reach the blastocyst stage during culture (Fry *et al.*, 1993; Smith *et al.*, 1994).

This level of vacuum was also shown to allow the follicular sample to be drawn into the sampling syringe against the vacuum with no air bubble contamination.

7.7 Summary of specifications

The need statements identified in this chapter through the trial of the sampling device in the clinical environment are summarised in Table 7.1 and the resulting specifications are summarised in Table 7.2.

Table 7.1 Summary of the nedd statements identified after using the follicular sampling system in the clinical environment.

Need Ref	Section Ref	Need statement	Rank
7.2	7.2	Sampler can be assembled quickly	R
7.3	7.3	Sampler does not leak air when vacuum is applied	I
7.4.2	7.4.2	The entire sampler is sterile	I
7.5	7.5	The sample is not drawn out of the sample container by the aspirating vacuum	R

Table 7.2 Summary of specifications identified after the sampling system was used in the clinical environment

Spec Ref	Need Ref	Metric	Unit	Value
Sp22	7.2	Maximum time to assemble sampler	min	10
Sp23	7.3	Maximum vacuum the sampler can tolerate	mmHg	300
Sp24	7.3	Maximum vacuum applied for aspiration	mmHg	120
Sp24	7.2 7.4.2	The entire sampler is sterile	Y/N	Y
Sp25	7.5	Maximum vacuum before sample is draw back out of the sample container	mmHg	300

7.8 Conclusions

Having had some initial difficulties, the sampling device has changed form and had its performance improved many aspects, such as aseptic handling, resistance to vacuum, weight and size. The test remains now to determine the predictive power of dissolved follicular oxygen on oocyte developmental competence.

Clinical testing of the device at reduced vacuum during oocyte aspiration could not be completed during the course of this study due to delays and difficulties in recruiting patient volunteers who were willing to test the procedure. It is suggested that this be carried out in future work using the modified sampling device with controlled vacuum levels.

Chapter 8. Conclusions

This project had two objectives:

- To develop a set of product specifications that will help to guide the design of a device for sampling follicular fluid. The samples will be used to determine the dissolved oxygen content of the follicular fluid.
- To develop a prototype device and use it in a fertility clinic.

The set of specifications to which the first objective alludes has been developed through time spent in the fertility clinics and through exploration of the nature of sampling and sampler materials. The need statements are summarised in table 8.1 and the specifications are summarised in table 8.2.

The prototype device to which the second objective alludes was developed, tried, and after a poor performance, improved. Use of the second iteration device unveiled more shortcomings in the design, which were also remedied.

The current device should be tested and used to demonstrate (or otherwise) the value of ovarian follicular fluid oxygen levels in characterising oocyte quality. Once this has been achieved it is suggested that the remainder to the product development process be carried forward, based on the specifications identified in this project. There are a number of possible product designs that may better meet the product specifications than the prototype developed in this work.

Table 8.1 A summary of the need statements developed throughout this project.

Need Ref	Section Ref	Need Statement	Rank*
		Sampler module	
3.5.3a	3.5.3	Sampler is not too large	S
3.5.3b	3.5.3	Sampler is not too heavy	S
3.5.4a	3.5.4	Sampler can be operated in confined space	S
3.5.4b	3.5.4	Sampler can be operated comfortably for more than 20 minutes	S
3.5.10a	3.5.10	A sample consists of fluid from one follicle only	I
3.5.5	3.5.5	Sampler does not increase aspiration kit volume	R
4.1.1a	4.1.1	Sampling does not deform the cumulus oophorus	R
4.1.1b	4.1.1	Sampling does not break the cumulus oophorus	R
4.1.1c	4.1.1	Sampling does not strip the cumulus	I
4.1.1d	4.1.1	Sampling does not damage the zona pellucida	I
4.1.1e	4.1.1	Sampling does not damage the oocyte	I
4.2.1a	4.2.1	Sampler materials are not known to be cytotoxic	R
4.2.1b	4.2.1	Sampler materials are not known to be embryotoxic	R
4.2.1c	4.2.1	Sampler materials are not known to be teratogenic	R
4.2.2a	4.2.2	Sampler can tolerate wash cycle	R
4.2.3a	4.2.3	Sampler passes mouse embryo testing	I
4.3a	4.3	Sampler tolerates sterilisation by steam	R
5.1.1	5.1.1	Sampler allows detection of blood contamination	I
5.1.2	5.1.2	Sample container material should not affect the sample DO	I
5.1.3	5.1.3	Bubbles in the sample do not affect the measured DO	I
7.2	7.2	Sampler can be assembled quickly	R
7.3	7.3	Sampler does not leak air when vacuum is applied	I
7.4.2	7.4.2	The entire sampler is sterile	I
		Sample container module	
3.5.8a	3.5.8	Sample container can contain a sample for an adequate length of time	R
3.5.9a	3.5.9	Sampler and sample container can tolerate temperature drop from 37°C to 0°C	S
3.5.9b	3.5.9	Sampler and sample container can tolerate being immersed in water	S
4.1.2a	4.1.2	Sampling does not raise the temperature	I
4.1.2b	4.1.2	Sampling does not reduce the temperature	R
4.1.3a	4.1.3	A sample can be screened for the presence of an oocyte	I
4.1.3b	4.1.3	An oocyte can be recovered from a sample	I
4.1.3c	4.1.3	A sample does not contain an oocyte	R
4.1.3d	4.1.3	The oocyte is not lost	R
4.2.1d	4.2.1	Sample container materials are not known to be cytotoxic	R
4.2.1e	4.2.1	Sample container materials are not known to be embryotoxic	R
4.2.1f	4.2.1	Sample container materials are not known to be teratogenic	R
4.2.2b	4.2.2	Sample container can tolerate wash cycle	R
4.2.3b	4.2.3	Sample container passes mouse embryo testing	I
4.3b	4.3	Sample container tolerates sterilisation by steam	R
5.2	5.2	Transferring the sample to the measuring equipment conforms to manufacturer's instructions	R
7.5	7.5	The sample is not drawn out of the sample container by the aspirating vacuum	R
		Oxygen measurement module	
3.5.6	3.5.6	Sample volume is small	S
3.5.8b	3.5.8	DO can be measured quickly	R
		Procedure module	
3.5.10b	3.5.10	A sample must be traceable to an oocyte	I
3.5.10c	3.5.10	A sample must be traceable to a follicular fluid	I
3.5.10d	3.5.10	A sample must be traceable to a patient	I
3.5.10e	3.5.10	The sample source information can be recorded	I

Table 8.2 Summary of clinical specifications

Spec. Ref	Need Refs	Metric	Unit	Value
Sp1	3.5.5	Sampler volume	ml	<1.5
Sp2	3.5.6	Volume of liquid removed	µl	<600
Sp3	3.5.3a 3.5.3b	Size of sampler	mm	120x70x30
Sp4	3.5.4a 3.5.4b	Number of people required to operate sampler	#	≤1
Sp5	3.5.8a 3.5.8b	Length of time from sample collection to end of oxygen determination	minutes	<60
Sp6	3.5.9a 3.5.9b	Temperature range sampler and sample container can tolerate	°C	<0-37
Sp7	3.5.9	Sampler and sample container can tolerate being immersed in water	Y/N	Y
Sp8	3.5.10a 3.5.10b 3.5.10c 3.5.10d	Number of times information regarding the source of a follicualr sample is lost	%	<2
Sp9	4.1.1a 4.1.1b 4.1.1c 4.1.1d 4.1.1e	Rate of OCCs showing shear effects	%	<2
Sp10	4.1.2a 4.1.2b	Temperature of sample	°C	33-39
Sp11	4.1.3a	Length of time required to find an oocyte in a sample	s	>30
Sp12	4.1.3b	Length of time required to recover an oocyte from a sample	s	>15
Sp13	4.2.1a 4.2.1d	Construction materials are not cytotoxic	Y/N	N
Sp14	4.2.1b 4.2.1e 4.2.3a 4.2.3b	Construction materials mouse embryo testing	Y/N	Y
Sp15	4.2.1c 4.2.1f	Construction materials are not teratogenic	Y/N	N
Sp16	4.2.2a 4.2.2b 4.3a 4.3b	Number of wash/sterilisation cycles until sampler or sample container fail	#	>100
Sp20	5.1.1 5.2.1 5.1.3	Maximum change in a sample's dissolved oxygen partial pressure	mmHg	<20
Sp21	5.2	The sample container is compatible with the oxygen measuring equipment	Y/N	Y
Sp22	7.2	Maximum time to assemble sampler	min	10
Sp23	7.3	Maximum vacuum the sampler can tolerate	mmHg	300
Sp24	7.3	Maximum vacuum applied for aspiration	mmHg	120
Sp24	7.2 7.4.2	The entire sampler is sterile	Y/N	Y
Sp25	7.5	Maximum vacuum before sample is draw back out of the sample container	mmHg	300

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Appendix 1. Calculation of the Effect of Addition of Blood to non-Blood Liquids on Dissolved Oxygen

```
%MATLAB Srcipt for Calculating Change in dissolved oxygen when blood is mixed
with a non-blood aqueous liquid
format long

global POFF; %pO2 of follicular fluid (mmHg).....ref Shalgi et al. (1972)
global POa;  %pO2 of Arterial Blood (mmHg).....ref Fournier p.90
global POv;  %pO2 of ovarian venous blood (mmHg).....ref Fraser(1973)
global Vt;   %Sample volume (mL)
global HCf;  %Henry's Constant for FF (mmHg.mL/g).....ref Fournier p.90
(0.74mmHg/uM)
global HCb;  %Henry's COnstant for Blood (mmHg.mL/g).....ref FOurnier p.90
(0.74mmHg/uM)
global Hcta; %Blood Haematocrit.....ref Mosby's p.1693 (adult female
range=37%-47%)
global Hctv;
global Chba; %Concentration of Haemoglobin in blood (mmol/mL).....ref Mosby's
p.1693 (adult female range=7.4-9.9mmol/mL)
global Chbv;
global n; %Hill Equation constant.....ref FOurnier p.90
global Pfifty;  %P50 of haemoglobin (mmHg).....ref Fourier p.90
global m;

global Sata; %Calculate Arterial Blood haemoglobin saturation
global Satv; %Calculate Venous blood Haemoglobin saturation

global Arterial;
global Venous;

%Set System inputs
POFF=54.3;   %pO2 of follicular fluid (mmHg).....ref Shalgi et al. (1972)
POa=95;   %pO2 of Arterial Blood (mmHg).....ref Fournier p.90
POv=88;   %pO2 of ovarian venous blood (mmHg).....ref Fraser(1973)
Vt=0.5;   %Sample volume (mL)
HCf=23125000;%Henry's Constant for FF (mmHg.mL/g).....ref Fournier p.90
(0.74mmHg/uM)
HCb=23125000;%Henry's COnstant for Blood (mmHg.mL/g).....ref FOurnier p.90
(0.74mmHg/uM)
Hcta=42; %Blood Haematocrit.....ref Mosby's p.1693 (adult female range=37%-
47%)
Hctv=37;
Chba=2.54e-3;%Concentration of Haemoglobin in blood (mmol/mL).....ref Mosby's
p.1693 (adult female range=7.4-9.9mmol/mL)
Chbv=1.905e-3;
n=2.34;   %Hill Equation constant.....ref FOurnier p.90
Pfifty=26;   %P50 of haemoglobin (mmHg).....ref Fourier p.90
```

```
%Calculate consequential inputs
Sata=(POa^n)/((Pfifty^n)+(POa^n)); %Calculate Arterial Blood haemoglobin
saturation
Satv=(POv^n)/((Pfifty^n)+(POv^n)); %Calculate Venous blood Haemoglobin
saturation

%Set up solution matrices
Arterial=ones(32,15);
Venous=ones(32,15);

for m=1:31
   Arterial(m,2)=(m-1)/50000; %Set volume of blood in arterial Matrix
   Venous(m,2)=(m-1)/50000;      %Set volume of blood in venous matrix
end
Arterial(32,2)=Vt;
Venous(32,2)=Vt;

Arterial(:,1)=Vt-Arterial(:,2);  %set volume of FF in arterial Matrix
Venous(:,1)=Vt-Venous(:,2);      %Set volume of FF in venous matrix
Arterial(:,15)=100*Arterial(:,2)./Vt;
Venous(:,15)=100*Venous(:,2)./Vt;

%Calculate terms in mass balance:
%Oxygen in blood + Oxygen in FF = Oxygne in mixture
%oxygen in plasma + oxygen in hb + oxygen in FF = oxygen dissolved in
(FF+plasma)+oxygen bound to haemoglobin

%Calculate terms on LHS (in)
Arterial(:,3)=Arterial(:,2)*(1-Hcta/100)*(POa/HCb); %Calculate amount of
Oxygen in arterial plasma (g)
Arterial(:,4)=Arterial(:,2)*Chba*Sata*4*32;   %Calculate amount of oxygen
bound to arterial haemoglobin (g)
Arterial(:,5)=Arterial(:,1)*POFF/HCf;            %Calculate amount of oxygne
dissolved in ff (g)
Arterial(:,6)=Arterial(:,3)+Arterial(:,4)+Arterial(:,5);%Calculate total
amount of oxygen going into sample (plasma+haem+ff)...(g)

Venous(:,3)=Venous(:,2)*(1-Hctv/100)*(POv/HCb);  %Calculate amount of Oxygen
in arterial plasma (g)
Venous(:,4)=Venous(:,2)*Chbv*Satv*4*32;   %Calculate amount of oxygen bound
to arterial haemoglobin (g)
Venous(:,5)=Venous(:,1)*POFF/HCf;         %Calculate amount of oxygne
dissolved in ff (g)
Venous(:,6)=Venous(:,3)+Venous(:,4)+Venous(:,5); %Calculate total amount of
oxygen going into sample (plasma+haem+ff)...(g)

%Iteratively solve problem
options=optimset('Display','off','tolX',1e-15);
for iter=1:32
   m=iter
   art=fminbnd('Oxy_art_balance',POFF,POa,options);
```

```
    Arterial(m,12)=art;

    ven=fminbnd('Oxy_ven_balance',POFF,POa,options);
    Venous(m,12)=ven;

iter=iter+1;
end


Arterial(:,13)=Arterial(:,6)-Arterial(:,11);
Arterial(:,14)=Arterial(:,12)*100/760;

Arterial;

Venous(:,13)=Venous(:,6)-Venous(:,11);
Venous(:,14)=Venous(:,12)*100/760;

Venous;

figure
plot(Venous(:,15),Venous(:,12),'--',Arterial(:,15),Arterial(:,12))
title('Effect of Mixing Venous (dash) and Arterial (solid) Blood and
Follicular Fluid on Sample Dissolved Oxygen')
xlabel('Amount of Blood in Sample (vol%)')
ylabel('Partial pressure of oxygen in sample (mmHg)')
Xlim([0,0.2])
grid
```

```
function diff_art=oxy_art_balance(art);

global POFF; %pO2 of follicular fluid (mmHg).....ref Shalgi et al. (1972)
global POa;  %pO2 of Arterial Blood (mmHg).....ref Fournier p.90
global POv;  %pO2 of ovarian venous blood (mmHg).....ref Fraser(1973)
global Vt;   %Sample volume (mL)
global HCf;  %Henry's Constant for FF (mmHg.mL/g).....ref Fournier p.90
(0.74mmHg/uM)
global HCb;  %Henry's COnstant for Blood (mmHg.mL/g).....ref FOurnier p.90
(0.74mmHg/uM)
global Hcta; %Blood Haematocrit.....ref Mosby's p.1693 (adult female
range=37%-47%)
global Chba; %Concentration of Haemoglobin in blood (mmol/mL).....ref Mosby's
p.1693 (adult female range=7.4-9.9mmol/mL)
global n; %Hill Equation constant.....ref FOurnier p.90
global Pfifty;  %P50 of haemoglobin (mmHg).....ref Fourier p.90
global m;

global Sata; %Calculate Arterial Blood haemoglobin saturation
global Satv; %Calculate Venous blood Haemoglobin saturation

global Arterial;
global Venous;

%Calculate RHS (mix)
Arterial(m,12)=art;

Arterial(m,7)=(Arterial(m,2).*Chba)./(Arterial(m,2)+Arterial(m,1));
   %Calcualte haemoglobin conc in mixture of blood and ff (mol/ml)
Arterial(m,8)=(Arterial(m,1)+(Arterial(m,2)*(1-Hcta/100))).*(art/HCb);
   %Calculate amount Dissloved oxygen in plasma+FF mix (g)
Arterial(m,9)=(art.^n)./((Pfifty^n)+art.^n);          %Calculate    haemoglobin
saturation in mix
Arterial(m,10)=Vt.*Arterial(m,7).*Arterial(m,9).*4*32;          %Calculate
amount of oxygen bound t haemoglobin in mix (g)
Arterial(m,11)=Arterial(m,8)+Arterial(m,10);      %Calculate  total  amunt  of
oxygen in mix (dissolved o2+bound O2)...(g)



diff_art=abs(Arterial(m,6)-Arterial(m,11));
```

```
function diff_ven=oxy_ven_balance(ven);

global POFF; %pO2 of follicular fluid (mmHg).....ref Shalgi et al. (1972)
global POa;  %pO2 of Arterial Blood (mmHg).....ref Fournier p.90
global POv;  %pO2 of ovarian venous blood (mmHg).....ref Fraser(1973)
global Vt;   %Sample volume (mL)
global HCf;  %Henry's Constant for FF (mmHg.mL/g).....ref Fournier p.90
(0.74mmHg/uM)
global HCb;  %Henry's COnstant for Blood (mmHg.mL/g).....ref FOurnier p.90
(0.74mmHg/uM)
global Hctv; %Blood Haematocrit.....ref Mosby's p.1693 (adult female
range=37%-47%)
global Chbv; %Concentration of Haemoglobin in blood (mmol/mL).....ref Mosby's
p.1693 (adult female range=7.4-9.9mmol/mL)
global n; %Hill Equation constant.....ref FOurnier p.90
global Pfifty;  %P50 of haemoglobin (mmHg).....ref Fourier p.90
global m;

global Sata; %Calculate Arterial Blood haemoglobin saturation
global Satv; %Calculate Venous blood Haemoglobin saturation

global Arterial;
global Venous;

%Calculate RHS (mix)
Venous(m,12)=ven;

Venous(m,7)=(Venous(m,2).*Chbv)./(Venous(m,2)+Venous(m,1)); %Calcualte
haemoglobin conc in mixture of blood and ff (mol/ml)
Venous(m,8)=(Venous(m,1)+(Venous(m,2)*(1-Hctv/100))).*(ven/HCb);  %Calculate
amount Dissloved oxygen in plasma+FF mix (g)
Venous(m,9)=(ven.^n)./((Pfifty^n)+ven.^n);       %Calculate       haemoglobin
saturation in mix
Venous(m,10)=Vt.*Venous(m,7).*Venous(m,9).*4*32;       %Calculate  amount  of
oxygen bound t haemoglobin in mix (g)
Venous(m,11)=Venous(m,8)+Venous(m,10);     %Calculate total amunt of oxygen in
mix (dissolved o2+bound O2)...(g)


diff_ven=abs(Venous(m,6)-Venous(m,11));
```

Appendix 2. MATLAB script for determination of the effect of air bubbles on liquid samples

```
%Script file for solving the equilibrium partial pressure of oxygen ina  lquid
sample exposed to an air bubble
clear all
format long

%declare Variable
global Vl;    %Volume of liquid sample (ml)
global pO2in;%initial oxygen partial pressure in liquid sample (m3)
global Hff;      %Henry's law constant for follicular fluid (pa.m3/mol)
global pO2atm;   %initial partial pressure of oxygen in air bubble (Pa)
global R;     %Ideal gas constant (kJ/mol.K)
global T;     %System temperature (K)
global m;
global solution;

%Assign variables
Vl=0.5*1e-6;         %Arbitrary value (m3)
pO2in=5065;             %5%atm (Pa)
Hff=98658.6;         %Fournier (???)
pO2atm=101300*0.21; %21% atmospheric @ 1bar (Pa)
R=8.3144;            %Just is (kJ/mol.K)
T=37+273;            %Body temperature (K)

%Set up solution matrix
solution=ones(10,5);
options=optimset('Display','off','TolX',1e-6);

for m=1:41
   solution(m,1)=(1e-6)*(m-1)/160; %set variable bubble volume 0.05=>0.5ml
   solution(m,3)=(Vl*pO2in/Hff)+((pO2atm*solution(m,1))/(R*T));%Calculate LHS,
o2in
   solution(m,2)=(solution(m,3)/((Vl/Hff)+(solution(m,1)/(R*T))));%calculate
pO2eq
   pO2eq=solution(m,2);
   solution(m,4)=(Vl*pO2eq/Hff)+((pO2eq*solution(m,1))/(R*T)); %Calculate RHS
   solution(m,5)=solution(m,3)-solution(m,4);                    %ensure that
LHS-RHS=0
end

volfract=solution(:,1)./Vl;
figure
plot(volfract,solution(:,2));
xlabel('Volume of air bubble/Volume of liquid');
ylabel('Equilibrium Partial Pressure (Pa)');
```

```
xlim=[0,0.5];
ylim=[0,20000];
grid
```