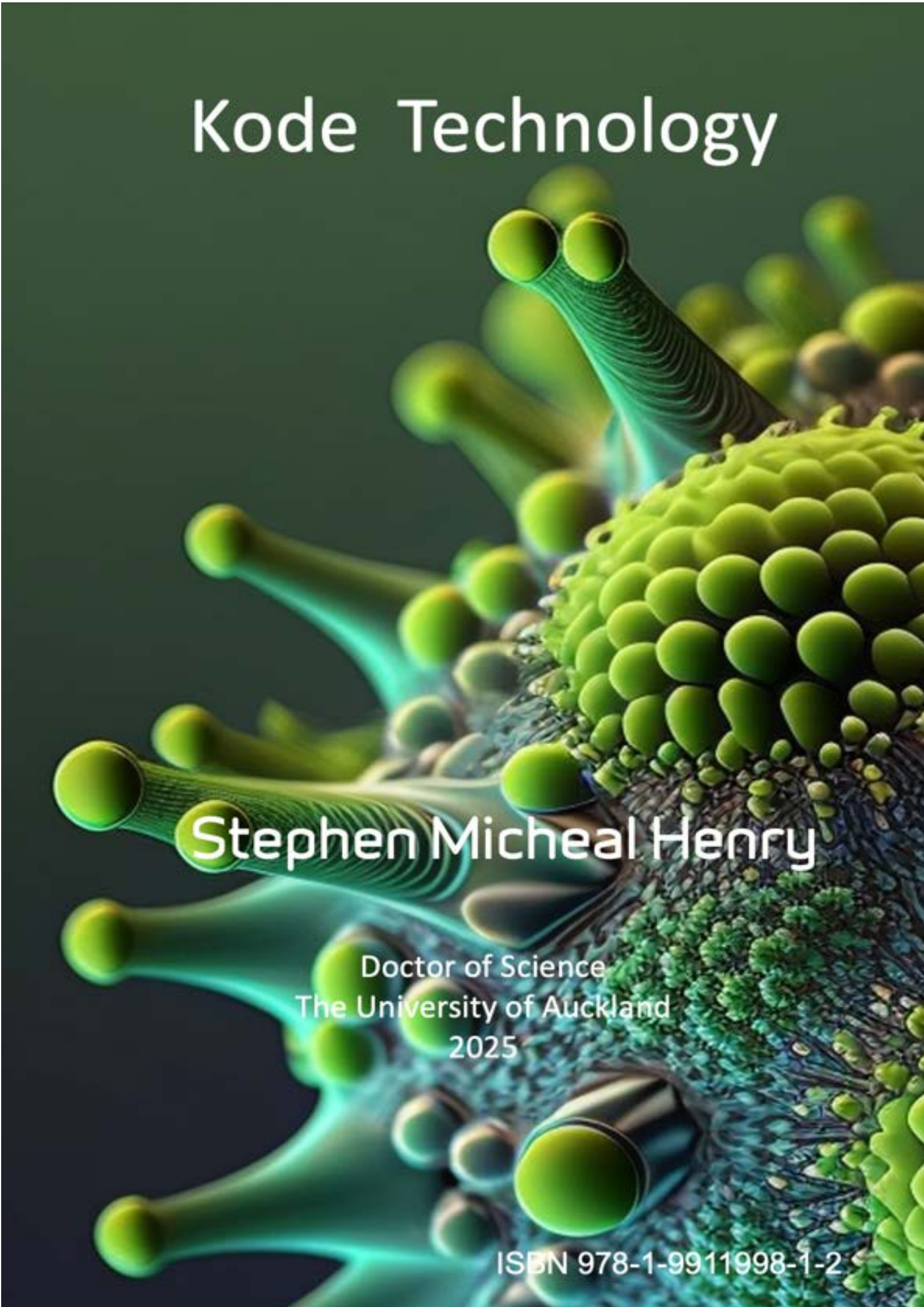
Kode Technology



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Doctor of Science
The University of Auckland
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Kode Technology

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Abstract

This DSc thesis is based on publications (journal publications, patents and the "Kode Technology Illustrated Technical Manual" eBook) associated with the author Stephen Henry's journey in developing Kode Technology. It starts with a pre-Kode Technology exploration of blood group related glycolipids, which evolved into the first generation use as natural glycolipids, before radically morphing into second generation nanotechnology synthetic glycolipids, which further evolved into third generation of Kode constructs, capable of modifying any biological or non-biological surface with almost any small molecule.

Today Kode Technology represents a nanotechnology platform of biosurface modification constructs (Function-Spacer-Lipids, FSLs or Kode constructs) which can enable the non-covalent attachment of essentially any small molecule onto any biological or non-biological surface. Kode Technology constructs although very sophisticated in their chemical composition are extremely low-tech with respect to their methods of use (koding); typically being as simple as bringing a surface (cell, virus, liposome or surface) in contact with a solution of Kode constructs for a short period of time, and the Kode constructs then spontaneously self-assemble into or onto the surface. The resultant controllable FSL construct modified (koded) surface coating is relatively stable, and although not permanent, is sufficient to enable potent biological activity, well within the biological time parameters.

Cells labelled with FSL constructs are known as kodecytes and have been used in a variety of different applications including; antibody diagnostics, quality control and training systems, visualisation, immobilisation, interactions with the immune system (including potential clinical applications), and in bespoke applications. Enveloped viruses labelled with FSL constructs have been used in a variety of similar applications. The power of Kode Technology is its ability (without affecting vitality and normal functionality) to rapidly impart on any living cell or enveloped virus, an additional function.

In addition to modification of biological entities, Kode constructs in solution phase and/or associated with lipid particles including liposomes are able to neutralise antibodies and toxins and adhere to almost any non-biological surface, including plastics, metals, rubber, glass, silicone, natural fibres, etc. Such koded surfaces have been used in a variety of applications including antibody diagnostics (where the Kode construct is inkjet printed onto the surface as alphanumeric characters), cell/virus surface adhesion and as an anti-microbial coating. With respect to opportunities for Kode Technology, as the functional head of a Kode construct can be almost anything, there is also an almost unlimited number of potential applications, and thus many new opportunities for the technology to change and reframe paradigms in biological methodology.

Although Kode Technology was co-invented by the author from the seeds of several decades of blood group glycolipid research, in reality the technology owes its existence to the extensive contributions from numerous colleagues and collaborators. This thesis, as well as extensively exploring the authors (and co-authors) contributions to Kode Technology, also includes the Kode Technology Illustrated Technical Manual Kode eBook https://natlib.govt.nz/records/50606977 which extensively covers all known published and unpublished Kode Technology data (up to 2022).

Synopsis

This DSc thesis documents the development and applications of Kode Technology, a nanotechnology-based platform for biosurface modification. The work evolved from early studies of blood group glycolipids into the creation of Function-Spacer-Lipid (FSL) constructs, also known as Kode constructs. Since almost any small molecule can serve as the functional head of a Kode construct, the range of applications is essentially unlimited, opening opportunities for paradigm shifts in biotechnology, diagnostics, and therapeutics. Kode Technology is a flexible, powerful, and broadly applicable platform capable of rapidly modifying biological and non-biological systems without compromising their function. Its simplicity, stability, and universality make it a promising tool for diagnostics, therapeutic development, and innovative surface engineering, with many yet-untapped opportunities.

Dedication

This thesis is dedicated with love and respect to my wife Bronwyn (who coincidentally shares the same birthday as Kode Technology), my large family and to my five colleagues and mentors (Professors Graeme Woodfield, Rafael Oriol, Bo Samuelsson, Roy Geddes and Nicolai Bovin), whose belief and faith in me created the foundations for the steps to invention and implementation that led to Kode Technology.

Mā te huruhuru ka rere te manu

Adorn the bird with feathers so it may soar

Preface and acknowledgements

Everything has a past, and that past guides and defines the outcome observed today, and as Carl Sagan (1980) once said "You have to know the past to understand the present". It is from this perspective, that this DSc thesis is written (which is part scientific autobiography of the author and part Kode Technology).

The thesis is written in the 3rd person, with the candidate simply referred to as the "author". Although 26th August 2003 is the birth date of Kode Technology, this thesis also journeys back in time to 1988, the author's glycolipid formative period, and also briefly further back to 1946 to its foundational origins, developed by others. These periods are relevant as they are where the concepts of Kode Technology were developed, and they influenced the path followed and ultimate realisation of the Kode Technology platform.

The APA referencing style has been used, primarily because it identifies the first author(s) name and year, which is useful when writing from an autobiographic perspective. To help identify those journal papers relating to the author's work, where the author was a contributor, those reference citations are in blue font (e.g. Henry *et al*., 1995c), while those references underlined in blue font (e.g. Henry *et al.*, 2018) are available as copies via Appendix 1. Additionally postgraduate student work cited and primary supervised by the author are reference indicated in blue font.

In the history of Kode Technology there is probably one journal paper which most influenced the work of the author, and that was the observations of Peter Sneath & Joan Sneath who in 1959 (Sneath & Sneath, 1959), observed that "Lewis substances in plasma absorb onto red cells" (Figure 1). It was these early observations which crystallised in the mind of the author, and later laid the conceptual foundations of what would eventually become known as Kode Technology. In 2009 when Kode Technology was still gaining form, the author wrote to Dr Peter Sneath (in the UK) and told him about Kode Technology, and how his early work had inspired the development of Kode Technology. The author also suggested that he considered him to be the grandfather of Kode Technology. Peter Sneath graciously responded with some personal photographs (Figure 1) and a letter which acknowledged the past and stated,

> *"What marvellous things have resulted from the early work of the three teams at the Lister Institute, led by Rob Race and Ruth Sanger, Walter Morgan and Winifred Watkins, and Arthur Mourant and Elizabeth Iken! … and certainly you may refer to me as grandfather of KODE technology, but if so my wife Joan is a grandmother!"* Peter Sneath, 2009

Like most science today it is rarely the result of an individual, and although the founding vision and concept of Kode Technology was that of the author, the founders of the Kode Technology platform were three individuals. Their overlapping skills were: the author, Stephen (Steve) Henry (entrepreneur and biologist), Nicolai V Bovin (biologist & chemist) and Alexander Tuzikov (chemist). Each of these individuals brought an essential skill-set and character to the journey; where Steve would dream up possible (and impossible) uses for the technology, and Nicolai, a rare speaker of the languages of both biologists and chemists, would then translate these ideas into rational chemical-speak, which would allow Alexander, a magician in chemistry to pull novel Kode constructs out of his hat (metaphorically that is). In reality, these actual roles were often blurred and very fluid (with many times Nicolai being the initiator of new Kode Technology applications), but overall it was essentially this combination of skills, and a strong 26 year active collaboration and friendship

that enabled the development of Kode Technology. There were of course many other individuals (too numerous to name here) who all contributed significant parts to the scientific development and commercialisation of Kode Technology.

Joan Sylvia Sneath
1928-2005

Peter Henry Andrews Sneath
(1923-2011)

ADSORPTION OF BLOOD-GROUP SUBSTANCES FROM SERUM ON TO RED CELLS

JOAN S. SNEATH B.Sc.
P. H. A. SNEATH M.A. M.B. B.Chir.

Medical Research Council
Blood Group Research Unit
Lister Institute of Preventive Medicine, London

National Institute for Medical Research
Mill Hill, London

Most of the blood-group antigens appear to be an integral part of the red cell. Work on transfused red cells and blood-group chimeras suggests that this is true of all the human blood-group systems except the Lewis system. The Lewis system is primarily a system of soluble tissue antigens (Grubb, 1951), and the antigens on the red cells are acquired by adsorption from the serum. The ABO system occupies an intermediate place for, like the Lewis system and unlike the others, ABH substances are present in water-soluble form in the secretions of about 75% of Caucasians. Two blood-group systems in animals show close parallels with the Lewis system, the J system of cattle and the R system of sheep. It is these three systems which will be discussed in this paper.

the information is still too scanty to say whether the following properties are always associated, but they have been so in our experience.

1. Some anti-Le^b sera are neutralized by the salivas of all ABH secretors, both of Le(a−b+) persons and of Le(a−b−) persons, but apparently never by salivas of Le(a+b−) persons. The Le^b red-cell character as defined by them shows marked epistatic suppression in the presence of the A_1 gene.

2. Other anti-Le^b sera are not neutralized by the saliva of Le(a−b−) ABH secretors, though they are neutralized by saliva of Le(a−b+) persons. They are inhibited by the saliva of a small proportion of Le(a+b−) persons, and show little epistasis with the A_1 gene.

We shall refer to type 1 and type 2 as anti-Le$^b_1$ and anti-Le$^b_2$ respectively, and the corresponding antigens as Le$^b_1$ and Le$^b_2$.

There are four well-recognized phenotypes in adults of groups O and A_2 (see Table I). A_1 and A_1B persons can

TABLE I. THE FOUR MAIN LEWIS PHENOTYPES AS DEFINED BY SECRETIONS ONLY

Red-cell antigens	Saliva antigens				Approximate frequencies in European adults (%)
	ABH	Le^a	Le$^b_1$	Le$^b_2$	
Le(a−b+)	+	+	+	+	70
Le(a+b−)	−	+	−	−	20
Le(a−b−)	+	−	+	−	8
Le(a−b−)	−	−	−	−	2

usually be allocated correctly to these four phenotypes by the use of anti-Le$^b_2$ and saliva tests, and the frequency of the phenotypes so determined is independent of the ABO groups.

The work of Andresen (1948a, 1948b), Jordal & Lyndrup (1952), Jordal (1956) and Speiser, Meznik-Schönbauer & Fürsten (1956) shows that new-born infants are Le(a−b−); the antigens appear on the red cells after some weeks, and then most children are Le(a+) and many are Le(a+b+).

Figure 1. Photographs of Joan and Peter Sneath (*circa* 1950) and the title and front page of their seminal reference (Sneath & Sneath,1959). A biography of Peter Sneath was published by the British Royal Society (Jones & Grant, 2013).

It is also important to appreciate that the development of Kode Technology was under the umbrella of a commercial enterprise, Kode Biotech Ltd, (founded by the author in 1996). The additional entrepreneurial skill set of Steve (although not a scientific one), was also important to the mix, as although it sometimes constrained the research directions (the need to be commercially viable), this very focussed approach and discipline also enabled the commercial funding and revenues essential to breathe life into the technology.

Despite its birth as a synthetic glycolipid in August 2003, Kode Technology today has grown to be much more than just synthetic glycolipids. However, to better understand the present we must also look at Kode Technology's history. Accordingly this thesis is organised into six chapters. **Chapter 1. Lewis Blood Group Glycolipids: Origins of Kode Technology** explores the history of the Lewis glycolipid blood group system including its relationships with the ABH and Secretor blood group systems and highlights the authors contributions to this field. **Chapter 2. Glycolipids – First Generation Kode Technology** briefly explores the predominantly unpublished pre-Kode Technology era in the form of natural glycolipids. **Chapter 3. Synthetic Glycolipids – Second Generation Kode Technology** explores the birth of Kode Technology as nanotechnology synthetic glycolipids, before morphing into the larger technology platform. **Chapter 4. CMG Spacers -Third Generation Kode Technology** explores the expansion of Kode Technology beyond glycans and introduces the CMG next generation FSL spacer. **Chapter 5. Kode Technology Application Themes.** This section is organised into Kode Technology FSL construct themes. Each theme discusses the *author's* published contributions to Kode Technology. The final theme discusses very briefly the role of the commercial entity Kode Biotech Limited. **Chapter 6. Concluding Remarks.** This brief chapter finishes with some concluding remarks on the future potential of Kode Technology.

Two appendices are provided, with **Appendix 1: Published Kode Technology Journal Papers** as a bookmarked/linked (e.g. Henry *et al.*, 2018) list for those published Kode Technology papers where the author was a contributor (excluding published journal abstracts and patents which are simply listed in the References). **Appendix 2: The Kode Technology Illustrated Technical Manual**, 1st edition, published in February 2023 (Henry *et al.*, 2023) is a comprehensive review on Kode Technology which presents and discusses all aspects Kode Technology including its scope, chemistry, features, methodology, applications, limitations and potential, and sources this information from all known Kode Technology publications (including all those in Appendix 1) and a large amount of as yet unpublished data.

The terms Kode and Kodecyte

Every new technology needs a name. Not only is it essential to differentiate the technology from that of others, but it is also important to create identity and ownership within an organisation. The origins of the words Kode and kodecyte were well documented, and are as below.

Origin of the word Kode

In 1999 while negotiating a license agreement with Ortho Clinical Diagnostics (OCD) for a quality control product, Dr Kathy Reis a senior scientist at OCD took the author out for a tour and an ice-cream at a café in Pennsylvania. There they started brainstorming for a name from the technology. The author wrote down their ideas on paper Doilies, where a series of names were proposed (Figure 2). The final selection was KODE

(where K was for Kiwi (from Kiwi Ingenuity the original name of the company), O was for Ortho (e.g. OCD), D (for designer) and E (for erythrocytes, being the proper name for red cells). Kathy & the author then signed the back of the doily to mark the occasion in a half-hearted belief that someday KODE would become something. Unfortunately, due to a last hour restructuring at OCD, the contract was not signed and the negotiations collapsed, and the meaning for O was lost. Over the following years the technology expanded use into all kinds of cells beyond just red cells and so the meaning for the E also became redundant. At that stage the acronym KODE became a pseudo-acronym, and also lost its capitalisation. Today Kode, more correctly known as Kode Technology, is simply the name of the technology which uses function-spacer-lipid constructs. With a name now for the technology, a short-time later the company changed its name from Kiwi Ingenuity Limited to Kode Biotech Limited.

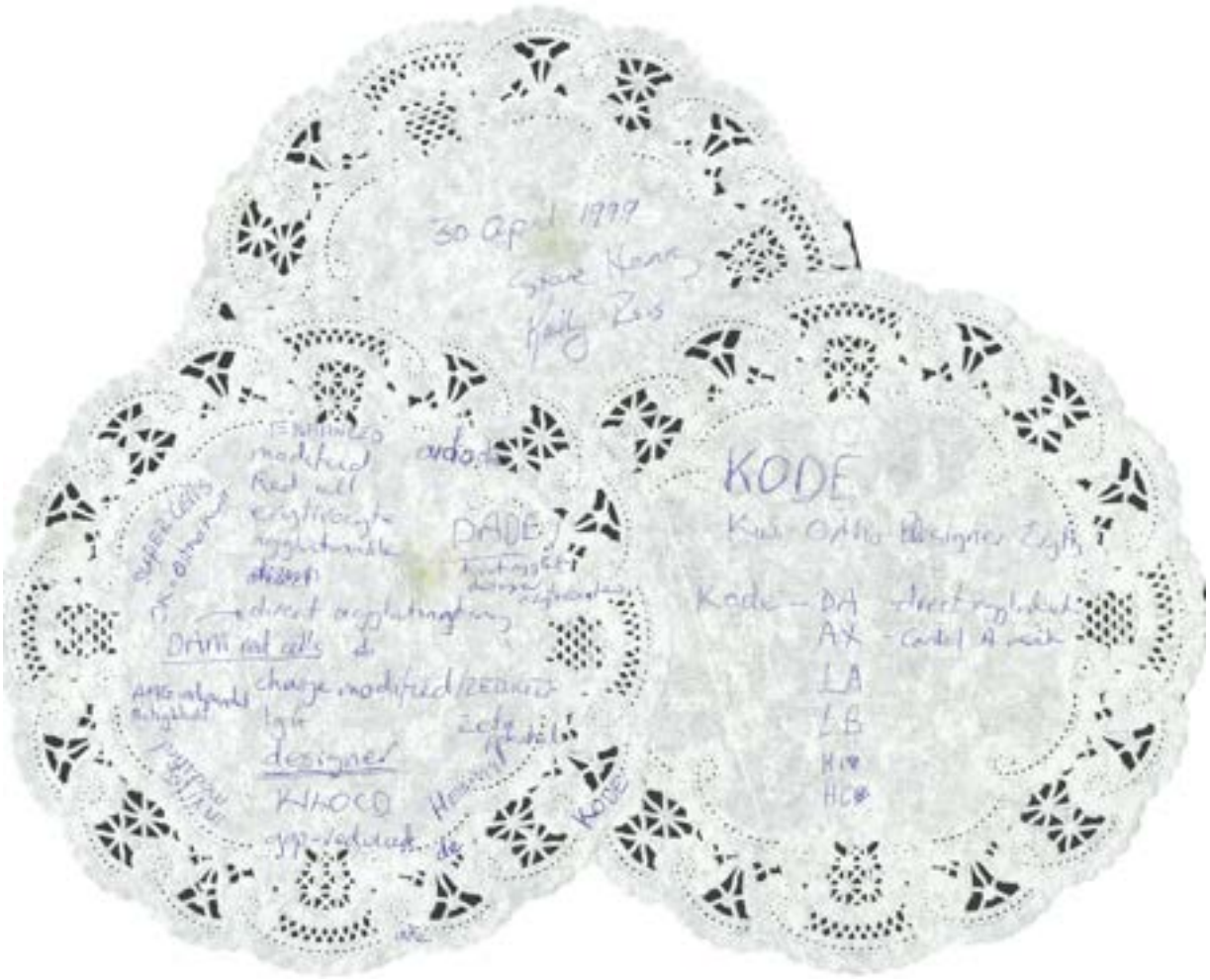

Figure 2. Photographs of the brainstorming doilies where the name Kode was originally conceived (30th April 1999). The original meaning was **K**iwi **O**rtho **D**esigner **E**rythrocytes, although this later became the pseudo-acronym Kode, which represents the technology. Fortunately for the author, Dr Kathy Reis kept the doilie's safe and kindly sent them to the author in 2012.

Origin of the word Kodecyte

Ten years later in 2009 while working closely with CSL, Damien Heathcote (Development and Quality Manager, Immunohematology Group) requested that we come up with a term to help describe the Kode Technology modified cells in their new products. The following is a verbatim summary of the two day email exchange, which resulted in developing the terminology for “kodecyte” and “koded”.

From: Damien.Heathcote@csl.com.au
Sent: Wednesday, 13 May 2009 3:44 PM
To: Stephen Henry
Subject: Word search

I need you two learned gents to help invent a new word for a cell that as KODE antigens/molecules attached. Useful site for greek/latin prefixes: http://www.kent.k12.wa.us/ksd/MA/resources/greek_and_latin_roots/transition.html
A quick look shows fer, port, syn, neo – all could apply. What can you come up with ?
I quite like neoerythrocyte but neocyte is in use I think.

From: Stephen Henry
Sent: Wednesday, 13 May 2009 4:07 PM
To: Heathcote, Damien
Subject: Re: Word search

mythrocyte – for modified erythrocyte

From: Damien.Heathcote@csl.com.au
Sent: Wednesday, 13 May 2009 4:11 PM
To: Stephen Henry
Subject: Re: Word search

Modicyte?

From: Stephen Henry
Sent: Wednesday, 13 May 2009 04:18 PM
To: Heathcote, Damien
Subject: Re: Word search

maybe

From: Damien.Heathcote@csl.com.au
Sent: Wednesday, 13 May 2009 4:20 PM
To: Stephen Henry
Subject: Word search

iCell or iCyte ? (I for inserted antigens)

From: Stephen Henry
Sent: Thursday, 13 May 2009 04:41 PM
To: Heathcote, Damien
Subject: Re: Word search

not bad – but very “Apple” - iCyte

From: Damien.Heathcote@csl.com.au
Sent: Wednesday, 13 May 2009 4:58 PM
To: Stephen Henry
Subject: Word search

agree

From: Stephen Henry
Sent: Thursday, 14 May 2009 9:55 PM
To: Heathcote, Damien
Subject: Re: Word search

what about kodacytes?

From: Stephen Henry
Sent: Thursday, 14 May 2009 9:56 PM
To: Heathcote, Damien
Subject: Re: Word search

kodecytes is probably better

From: Stephen Henry
Sent: Thursday, 14 May 2009 10:02 PM
To: Heathcote, Damien

Subject: Re: Word search
kodecytes – pronounced ko dee cytes

> **From:** Damien.Heathcote@csl.com.au
> **Sent:** Thursday, 14 May 2009 10:05 PM
> **To:** Stephen Henry
> **Subject:** Word search
> works for me

From: Stephen Henry
Sent: Thursday, 14 May 2009 10:19 PM
To: Zak Henry
Subject: new term
Hi Zak, Please create your dictionary thing for the following word – some suggestions as follows – we will use it in our videos and probably the CSL one
Kodecyte – a cell modified with KODE(tm) cell surface engineering technology.
Pronunciation – sounds like ko de site (use proper dictionary terms and layout)
Origin: <Gk kyto-, comb. form of kytos container, receptacle, body + KODE(tm) cell surface engineering technology

From: Stephen Henry [mailto: Stephen.henry@aut.ac.nz]
Sent: Thursday, 18 May 2009 02:32 PM
To: Zak Henry
Subject: Re:kodecytes definition
Hi Zak – I think pronunciation should be "code a site" not code e site
plus we will introduce the concept of "koded" a cell surface engineering process of coating a living cell with KODE(tm) function-spacer-lipid (FSL) constructs, a process which creates kodecytes

> **From:** Zak Henry [mailto: zak.henry@aut.ac.nz]
> **Sent:** Thursday, 18 May 2009 03:41 PM
> **To:** Stephen Henry
> **Subject:** fixed
> ko•de•cyte
> **noun,** a cell modified with KODE™ cell surface engineering technology
> ORIGIN from KODE™ - cell surface engineering technology + Greek *Kytos 'vessel, receptacle, body'*

Authorship statement and co-authorship declarations

Kode Technology was developed during the mid to late academic years of the author, and at this stage of an academic career most practical work is undertaken by postgraduate students and collaborators (however, the author as a specialist in serology usually read most manual serologic reactions). The journal papers in this DSc thesis therefore contain significant contributions of many students and international collaborators. Additionally the author's Authorship order policy is

> "as principal or senior investigator I usually assume the last author position or if co-authoring with other principal/senior investigators one of last authorship positions, with the first authorship positions usually being assigned to the researchers/students who undertook the primary practical work."

The extent of contribution of the author and collaborators has been documented and declared in the Co-authorship Declarations relating to the journal articles listed in Appendix 1 (and the eBook included in this thesis). This declaration document is available as a separate pdf document.

Acknowledgements

This thesis has been compiled around publications related to contributions of the author associated with the development of Kode Technology (including journal articles, journal published meeting abstracts, patents, and postgraduate student research supervised by the author). There were many contributors (too numerous to name individually) to this story, including colleagues, collaborators, co-authors, postgraduate students, research and support staff who have taught, mentored and worked with me over four decades of my scientific career. It must be appreciated this account is being told from the bias and through the eyes of the author, and the research contribution of others in many instances has only been given a fleeting mention (or worse not included), yet their contribution no matter how small was still part of the whole development of the technology. I hope you may forgive my oversights, and instead remember with fondness the value of your contribution. To all those individuals named and unnamed who have contributed I sincerely thank you. However, one individual stands out above the rest, and deserves mention by name, and that is my good friend and colleague Professor Nicolai Bovin, (co-inventor of Kode Technology) to whom I owe my deepest thanks.

I will simply conclude with the Māori proverb “Mā te huruhuru ka rere te manu”, which literally translates to “Adorn the bird with feathers so it may soar”, so thank you my friends and colleagues for all the feathers needed to allow me and Kode Technology to soar.

Cover image

The artistic biological surface image on the thesis cover is generated by Artificial Intelligence (AI) using a “text to image” algorithm (https://www.midjourney.com). Midjourney grants a CC BY-NC 4.0 license to use this image.

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Chapter 1. Lewis Blood Group Glycolipids: Origins of Kode Technology

Kode Technology today is much more than just synthetic glycolipids. However, so we can better understand the present, we must first look at the Lewis blood group system and glycolipid origins of Kode Technology.

Glycolipid blood group ancestors

The work of Sneath & Sneath (Sneath & Sneath, 1959) although the most influential to the author, was not the real beginning. But where to start, as there are clearly many historical scientific discovery events, upon which Kode Technology depends. However, a reasonable start of the journey is probably the discovery of the Lewis blood group system in 1946 (Mourant, 1946), and its later recognition as being a glycosphingolipid that was adsorbed by red cells from the plasma, which probably marked the beginning and pre-history of Kode Technology, particularly as the Lewis blood group system pre-occupied the author's formative years (1979-1995) in research.

The Lewis blood group system in the form of the Lewis a (Le^a) antigen was first described by Mourant (1946) and 2 years later was followed by the discovery of it antithetical partner Le^b first described in 1948 by Andresen (1948). However, the Lewis blood group antigens Le^a & Le^b were not the same as other antithetical blood group systems, and the initial basis for this was revealed when Grubb (1948; 1951) and Brendemoen (1951) independently observed that an individual's Lewis blood group phenotype was dependent on secretion of blood group ABH and Lewis antigens in saliva (see below for detailed explanation).

Overview of the genetics and biochemistry of the Lewis blood group system

There are many textbooks and journal papers related to ABH, Lewis and Secretor blood group systems. However, in order to highlight the author's contribution to the field, references used will be both key references and preferentially those references of the author. For a more comprehensive and balanced list of citations, the reader is referred to the references within the papers cited.

Today it is well established that this relationship is caused by a complex interaction of three and sometimes four different blood group glycosyltransferases, as reviewed in (Oriol *et al.,* 1986; Clausen *et al.,* 1989; Henry *et al.,* 1995a; 1996a; 1996e; Henry, 2001) and summarised in Figure 3. In brief, and with respect to Lewis and related saliva phenotypes, an individual inherits four genetically different and independent blood genes (*H*, *ABO*, *Lewis* and *secretor*). Each of these genes has at least one recessive allele (usually represented by lower-case letters) which result in an absent or inactive glycosyltransferase. For example in the H blood group system the dominant allele is *H* while the recessive allele is *h*, and similarly for Lewis and secretor (e.g. *Le/le, Se/se*), although for the ABO blood group system the recessive allele is *O* (originally meaning null). However, this is now known to be an over simplification, and many different alleles can also result in partially active glycosyltransferases, which in turn will influence the observed phenotype (Henry *et al.,* 1995a; 1996a). For the sake of accuracy, it should also be noted the recessive alleles usually encode for a protein, but it is the encoded glycosyltransferase protein which is inactive. Several of these new, inactive and partially active blood group related glycosyltransferases were co-discovered by the author (Henry *et al.,* 1996b; 1996c; 1996d; Larson, *et al*., 1996; Fernandez-Mateos *et al.,* 1998; Svensson *et al.,* 2013).

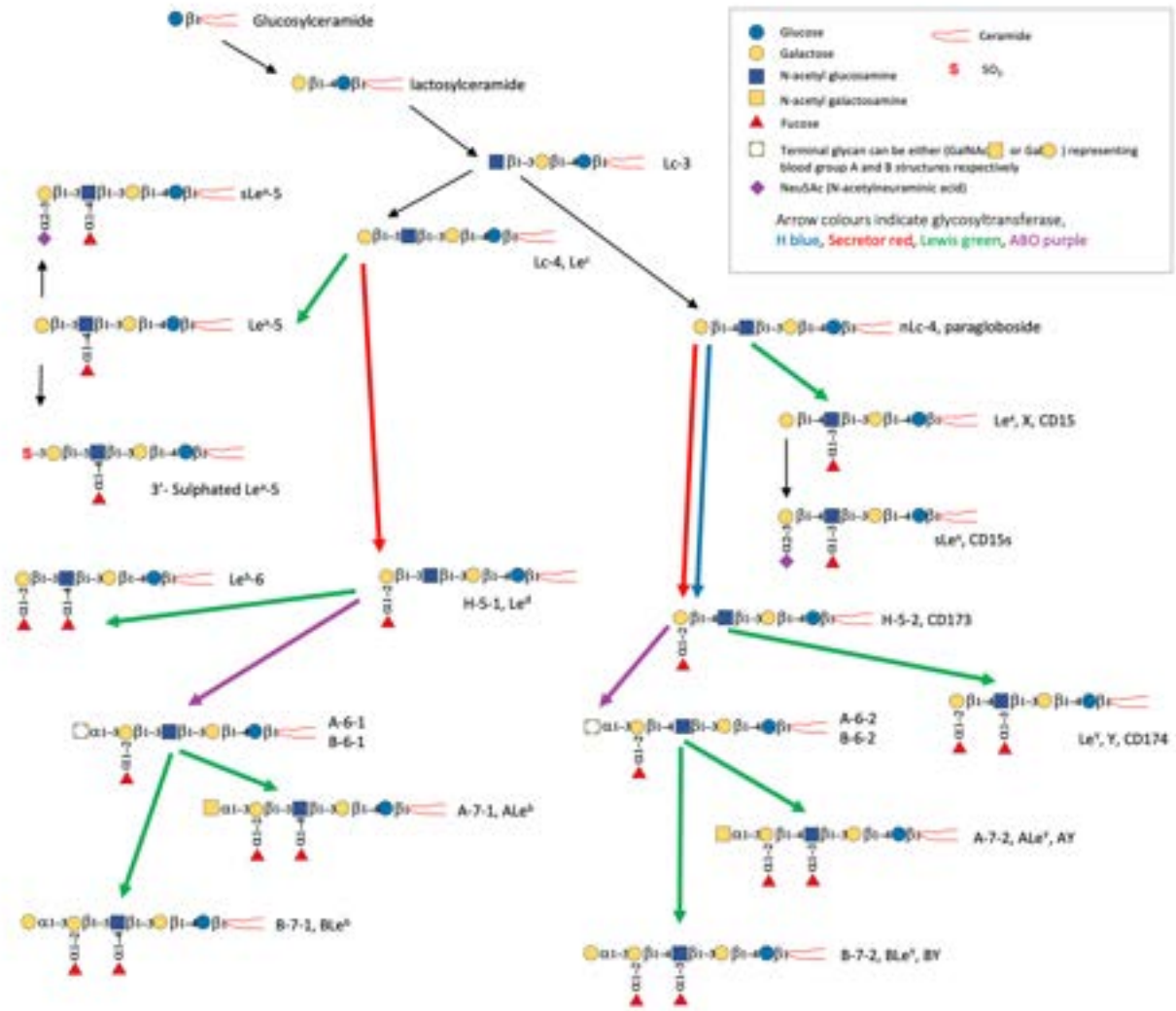

Figure 3. Overview of the biosynthetic pathways for ABH, Lewis and related antigens. Adapted from Henry *et al.*, 2023. Coloured lines indicate the action of the different glycosyltransferases, with blue the H α1-2 fucosyltransferase (FUT1), red the Secretor α1-2 fucosyltransferase (FUT2), green the Lewis α1-3/4 fucosyltransferase (FUT3) and purple the ABO α1-3 glycosyltransferases.

With respect to the Lewis (and related ABH) biosynthesis chemistry a number of different glycosyltransferase genes must be inherited as reviewed in (Oriol *et al.,* 1986; Henry *et al.,* 1995a; 1996a; Henry, 2001). In order to make ABO antigens on red cells an individual must first inherit an *H* gene to make the ABO system antigens. The *H* gene encodes the H enzyme which creates the H antigen, the requisite precursor for A & B antigens. The glycosyltransferases encoded by the immunodominant *A* and *B* genes are able to modify H antigen into an A and B antigen by the addition of a GalNAcα or Galα residue, respectively. In contrast, the *O* allele (which generally represents any genetic mutations in *A* & *B* alleles which results in an inactive enzyme) leaves the H antigen unmodified, and so H antigen by default is recognised as blood group O. A large number of *A* and *B* variant alleles exist, each of which encodes for lesser and lesser efficient glycotransferases resulting in decreasing levels of A or B antigens on red cells (representing phenotypes like A_1, A_2, A_3, A_x, A_{el} etc.) (Oriol *et al.,* 1986; Clausen *et al.,* 1989; Svensson *et al.,* 2005; 2009; 2011; n.d.). The

H antigen made by the H enzyme plays no part in the synthesis of type 1 Lewis antigens (Le^a, Le^b), but can have a role with type 2 antigens (Le^X, Le^Y). Individuals lacking the H antigen (e.g. Bombay/ParaBombay phenotype) will have normal expression of Lewis enzyme although their Se gene may be missing (Fernandez-Mateos *et al.,* 1998, Costache *et al.,* 1997).

However, there is more than one type of H antigen, and more than one H antigen making enzyme. The predominant H antigen of the red cell is created by the H enzyme (FUT1, fucosyltransferase 1) and is type 2 H antigen (that is it is based on the type 2 precursor Galβ1-4GlcNAc1-R (which is part of the Ii blood group system)). It is of note that the H enzyme cannot make H type 1(Oriol *et al.,* 1986).

The second H antigen making enzyme, is genetically independent of H and is known as the Se enzyme (FUT2, fucosyltransferase 2). The Se enzyme can make H antigen from both type 1 (Galβ1-3GlcNAcβ1-R) and type 2 precursors, but as it resides in epithelial cells (in contrast to the H enzyme which is present in mesenchymal (blood cell-forming) tissues) it does not make the H type 2 antigens intrinsic to the red cell. Instead it makes the H antigens present in epithelial secretory cells, and thus is responsible for the type 1 and type 2 H antigen in secretions, upon which A & B antigens can be formed (and hence the Secretor origin of its name). There is, however, a further enzyme in the secreter system called Se^w, discovered by the author (Henry *et al.,* 1995a; 1996b) and others (Yu *et al.,* 1995), and because it is less efficient than the normal Se fucosyltransferase (Henry *et al.,* 1995a) it changes the ratio of enzymatic activity between Le & Se, and results in the salivary partial secretor and blood group phenotype Le(a+b+), (see below, Phenotypes of Lewis). There are of course many *se* alleles which result in active enzymes (Kelly *et al.,* 1995; Henry *et al.,* 1996b; Svensson *et al.,* 2000)

The Lewis system which is completely independent of the H and Se systems also make a fucosyltransferase (FUT3), but one which does not make H antigen. The enzyme has a broad range and uses a large number of different precursor including type 1 and type 2 precursors (in competition with H & Se enzymes); H type 1 and H type 2 antigens (in competition with A & B enzymes); and type 1 A & B antigens (no competition), to make its range of Lewis antigens including Le^a, Le^b, ALe^b, BLe^b. Additionally the enzyme can use the type 2 family of precursor (along with other fucosyltransferases) and making X (Le^x), Y (Le^y), ALe^Y (AY) and BLe^y (BY) antigens (Oriol *et al.,* 1986; Henry *et al.,* 1995a). The blood group phenotypes and amount of each antigen resulting from these biosynthesis interactions depends on the individuals genotype (Oriol *et al.,* 1986; Henry *et al.,* 1995a; 1996a).

Phenotypes of Lewis

There are four major phenotypes of the Lewis blood group system; being Le(a-b-), Le(a+b-), Le(a-b+) and Le(a+b+), although most Caucasian dominated countries usually only consider the first three (Henry *et al.,* 1995a). Unlike other blood group systems Lewis phenotypes are not encoded by two antithetical alleles, i.e. there are not Le^a and Le^b genes or enzymes. Instead a complex interplay between a range of enzymes from the three independent systems results in the observable phenotypes (Figure 3).

The Le(a-b-) phenotype, with a frequency of about 10% in Caucasians is a consequence of inheritance of two recessive *le* alleles (e.g. *le/le*). However as Lewis is independent of Secretor, Lewis negative individuals can be either secretors or non-secretors (or partial secretors) of ABH substances. Lewis negative individuals

do however have trace amounts of Lewis antigens, but made by non-Lewis enzymes (Henry *et al.,* 1995b; 1994a; 1994c; Le Pendu & Henry, 2002; Ångström *et al.,* 2004).

The Le(a+b-) phenotype (which is about 20% frequent in Caucasians) is a consequence of inheritance of a Lewis positive genotype (e.g. *Le/Le or Le/le*) for a functional Lewis fucosyltransferase that is able to modify the type 1 precursor into Le^a antigen. The type 1 precursor is also the same precursor used by the Se fucosyltransferase to make H type 1 antigen, which if modified by the Lewis fucosyltransferase would instead result instead in Le^b antigen. Therefore for a cell to have Le^a and no Le^b then the Se enzyme must be absent (secretor genotype would typically be *se/se*; Kelly *et al.,* 1995*)*, and as a consequence cells of the Le(a+b-) phenotype are typically non-secretors (however note the caveat that the Le(a+b+) is frequently mistyped as Le(a+b-) due to it lower levels of Le^b antigen (Henry *et al.,* 1995a; 1989a; 1989b; 1988; 1996a; 1996e). Secretion of Lewis substance is independent of secretor status (which only controls ABH substances) and so Le(a+b-) individuals secrete Le^a in their saliva (Henry *et al.,* 1990; 1995a). It is also of note that the Le^a antigen cannot be modified by the Se fucosyltransferase in Le^b.

The Le(a-b+) phenotype (which is about 70% frequent in Caucasians) is a consequence of inheritance of a Lewis positive genotype (e.g. *Le/Le or Le/le*) for a functional Lewis fucosyltransferase that is able to modify the H type 1 precursor into Le^b antigen. However, the Le(a-b+) red cell phenotype is actually an artefact of serological reagent formulation, as all Le(a-b+) individuals have low levels of Le^a antigens, but phenotyping reagents have been tuned to not detect them (Henry *et al.,* 1995a; 1989a; 1989b). The Le(a-b+) phenotype is a result of an individual inheriting both a Lewis positive genotype (*Le/Le or Le/le*) and a Secretor positive genotype (*Se/Se or Se/se*) resulting in functional fucosyltransferases. These two enzymes are present together. The Se enzyme is very efficient at converting type 1 precursor into H type 1, while the Le enzyme is very much less efficient (Henry, 1996a; Oriol *et al*., 1986). As a consequence most type 1 precursor becomes H type 1 and only a small amount become Le^a. However the persistent Le enzyme (in the absence of A or B glycosyltransferase) is able to convert most of the H type 1 antigen into Le^b. The direct consequence is large amounts of Le^b are made and small amounts of Le^a, which using specifically serological formulated reagents will give the Le(a-b+) phenotype. Biosynthesis is more complicated in individuals where A or B enzymes are also present (Figure 3). These enzymes will outcompete the Lewis enzyme for the H type 1 precursor and convert it into A or B type 1 antigen. However, once again the Lewis enzyme gets the final say, and modifies most A and B type 1 antigens into the Lewis antigens ALe^b and BLe^b, respectively. This time the phenotype is still Le(a-b+) but the Le^b reactions are typically less than in group O individuals, due to sharing H type 1 with the ABO glycosyltransferase (and may be mistyped). Secretion of Lewis substance is independent of secretor status and so Le(a-b+) individuals secrete both Le^a and Le^b in their saliva, along with ABH substances that correlate with the ABO blood type.

With the Le(a+b+) phenotype biosynthesis becomes much more complicated (Henry *et al.,* 1988; 1989b; 1990b; 1993b; 1993b; 1994b; 1994c; 1996c; Henry, 1989a; 1990a; 1993a; 1995c; 1996e) as the Se enzymes loses its dominance over the Le enzyme, and they become equally efficient at competing for type 1 precursor. The net result is there is more Le^a and less Le^b antigen and less salivary ABH substances (the partial secretor phenotype). Secretion of the type of salivary substance is similar to the Le(a-b+) phenotype although the ratio of antigens are different with more Le^a and less Le^b and less ABH substances (because Le^a consumes

type 1 precursor preventing it from being made into the H type 1 and subsequently Le^b and/or ABH substances).

Prior to 1980 the Le(a+b+) phenotype was considered a very rare blood group system (Mourant *et al*., 1976), observed occasionally in Caucasians and more frequently in Aborigines (Boettcher & Kenny, 1971), Asians (Sturgeon & Arcilla, 1970) and Polynesians (Simmons & Graydon, 1951). Relatively little was known about the phenotype other than it was associated with a weak ABH secretor phenotype (Boettcher & Kenny, 1971).

The Le(a+b+) phenotype was infrequently observed at the NZ Blood Service, (where the author was working, under the guidance of Dr Graeme Woodfield, Figure 4), and was largely undocumented, so the author in 1987 decided to simply document its frequency (Henry *et al.,* 1988).

This opened Pandora's box and decades of research into this unusual phenotype and the world of glycolipids (Henry *et al.,* 1988; 1989a; 1989b; 1990b) genetics (Henry *et al.,* 1996a; 1996b; 1996c), and biochemistry (Henry *et al.,* 1993b; 1994b; 1994c; 1995c; Henry, 1993a; 1990a), finally culminating in the basis of Le(a+b+) being resolved; and proven to be due to a Se^w enzyme. A list of the author's publications related to Lewis and related blood group research (excluding glycolipids listed in Table 4) are listed in Table 1.

Graeme Woodfield
circa 1980

Figure 4. Dr Graeme Woodfield, CNZM, Medical Director (1976-1998) of the NZ Blood Transfusion Service. Dr Woodfield was the first research mentor for the author and enabled and encouraged him into the world of blood group research.

Table 1. Titles of publications in chronological order of author contributions related to **Lewis and related blood group research**. Journal published conference/meeting abstracts are not listed or referenced. This Table is excluding glycolipid research which is listed in Table 4 and Kode Technology related research which is listed in Tables 10, 11, 12.

Reference	Article Title
Henry *et al.,* 1988	The Le(a+b+) phenotype in Polynesians
Henry, 1989a	The serology and genetics of the Le(a+b+) phenotype in Polynesians
Henry *et al.,* 1989b	Investigation of Polynesian Lewis phenotypes. Variability in detection of Lewis antigens by monoclonal, goat and human antisera
Henry, 1990a	Immunochemical and biochemical studies of the Polynesian Lewis system
Henry *et al.,* 1990b	Investigation of Polynesian Lewis phenotypes. Evidence of a weak secretor phenotype
Candelier *et al.,* 1993c	α-3-fucosyltransferases and their glycoconjugate antigen products in developing human kidney
Henry *et al.,* 1995a	Lewis histo-blood group system and associated secretory phenotypes
Henry *et al.,* 1996d	A second nonsecretor allele of the blood group α(1,2)fucosyltransferase gene (FUT2)
Henry *et al.,*1996a	Review: phenotyping for Lewis and Secretor histo-blood group antigens
Henry *et al.,* 1996b	Homozygous expression of a missense mutation at nucleotide 385 in the FUT2 gene associates with the Le(a+b+) partial-secretor phenotype in an Indonesian family
Henry, 1996e	Le(a+b+); phenotype and genotype
Henry *et al.,* 1996c	Molecular basis for erythrocyte Le(a+b+) and the salivary ABH partial-secretor phenotypes. Expression of a FUT2 secretor allele with an A→T mutation at nucleotide 385 correlates with reduced α(1,2)fucosyltransferase activity
Larson *et al.,* 1996	Identification of a new plasma α(1,3)fucosyltransferase (FUT6) allele requires an extended genotyping strategy
Fernandez *et al.,* 1998	Point mutations and deletion responsible for Bombay H null and Reunion H weak blood groups
Costache *et al.,* 1997	Evolution of fucosyltransferase genes in vertebrates
Gustavsson *et al.,* 1999	Le^b glycolipids in the lumen of the gastrointestinal tract of rats do not enter the plasma compartment
Svensson *et al.,* 2000	Secretor genotyping for A385T, G428A, C571T, C628T, 685delTGG, G849A and other mutations from a single PCR reaction
Henry, 2001	Molecular diversity in the biosynthesis of GI tract glycoconjugates. A blood group related chart of microorganism receptors
Le Pendu & Henry 2002	Immunochemical, immunohistological and serological analysis of monoclonal antibodies with carbohydrates. Coordinators Report.
Henry, 2014	FUT2 mutation A385T does not result in a non-secretor allele (letter)
Svensson, *et al.,* (n.d.)	Blood Group ABO and its Variants. http://www.glycopedia.eu/e-chapters/blood-group-abo-and-its-variants/article/introduction-to-blood-group

A final comment on the Le(a+b+) phenotype. Despite the extensive research undertaken by the author and others at the time, the phenotype has regressed in being understood. Although in reality the Le(a+b+) phenotype on a population basis is probably the second most frequent Lewis positive phenotype in man, it is still considered uncommon, an error perpetuated by the Caucasian paradigm and where reagent manufacturers continue to formulate Lewis phenotyping reagents to force the Le(a+b+) phenotype into either the Le(a+b-) or Le(a-b+) phenotypes (Henry *et al.,* 1995a). Thus the Lewis blood group still has the dubious honour of probably being the most inaccurately phenotyped blood group system; and with recent papers (Guo *et al.,* 2017, including references therein) inaccurately citing the Se^w A385T mutation as a non-secretor

allele, which it is not (Henry, 2014), it has little chance of redemption. Furthermore, although the Lewis blood group system probably has some biological significance with respect to microbiology (Henry *et al.,* 1995a; Henry, 2001), its lack of any transfusion clinical significance (Waheed *et al.,* 1981, Henry *et al.,* 1995a) means there is no pressure to accurately determine Lewis phenotypes, and unless accurate phenotypes can be determined the ability to associate Lewis phenotypes with disease is impossible.

Lewis & related glycolipids

Not only are Lewis antigens biosynthetically different from most other blood group antigens, but the way in which they become part of the red cell antigens repertoire is also very different. Typically when the red cell is being synthesised its antigens, many of which carry membrane functions, are assembled with the cell. However, Lewis antigens are not synthesised with the red cell, and are instead synthesised by epithelial cells which shed them into the plasma in the form of glycolipids, where they associate with HDLs and LDLs, from where they are secondarily acquired by red cells (Oriol *et al.,* 1986; Henry *et al.,* 1995a). In contrast, the Lewis antigens of epithelial cells are synthesised by the cell. It should also be noted that Lewis antigens (as well and ABH antigens) also exist as glycoproteins in bodily secretors including saliva (Slomiany & Slomiany, 1978; Oriol *et al.,* 1986; Henry *et al.,* 1995a).

The first evidence that antigens on blood cells could be secondarily acquired from the plasma was made in 1949 in cattle, where it was found that incubation of J positive serum with J negative red cells would make them J positive (Stormont, 1949). Six years later Rendel reported a similar phenomenon for the R blood type of sheep (Rendel *et al;* 1954). In 1957 Nicholas et al (Nicholas *et al*., 1957) observed that the A/O mixed cell populations of chimeric twins, only had a single but different Lewis phenotype, and that it matched the ABH/Lewis substance secretor status of the individual. This was proof that the Lewis blood type was not haemopoietic (made by the red cells) but was instead acquired from their plasma, and that it evenly labelled all cells. A few years later in 1959 Sneath and Sneath proved that plasma in humans was the source of Lewis antigens on red cells by secondarily adsorbing them onto antigen negative cells *in vitro* (Sneath & Sneath, 1959). Additionally in 1962 Renton & Hancock (Renton & Hancock, 1962) showed the phenomenon for A and B antigens as consequence of transfusion (which is due to type 1 A and B glycolipids).

The identity of these plasma substances which had the ability to antigen modified blood cells over the next decade were identified as being glycolipids in both humans (Mäkelä *et al.,* 1967; Marcus & Cass, 1969; Tilley *et al*., 1975; Hanfland & Egli,1975; Hanfland et al., 1978) and animals (Stone, 1962; Thiele & Koch, 1970).

This ability of Lewis glycolipids to label red cells became the foundational basis of Kode Technology, and from 1996 – 2000 the author explored this phenomenon both *in vitro* and *in vivo* (in laboratory animals infused with purified glycolipids), with most of the work going unpublished (although some data was included in a patent application, WO 03/034074A1, Figure 7).

A couple of examples of these results are in Tables 2 and 3 (and also documented in the Kode Technology Illustrated Technical Manual - Appendix 2, Henry *et al*., 2023)

Table 2. An example of the *in vitro* uptake by Le(a-b-) and Le(a+b-) red cells of purified Le^b glycolipids. Cells were suspended in Le(a-b-) plasma and incubated for 2 h at 37℃. Results show uptake of Le^b glycolipids by the red cells, and the minimal consequence of increasing the ratio of red cells to glycolipids suggesting a serological equilibrium was being reached between the plasma and red cells.

Red cell phenotype	Le^b : RBC (v/v)	Antiserum reactions	
		-Le^a	-Le^b
Le(a-b-) before insertion	0	-	-
	1:2	-	+
	1:3	-	++
Le(a-b+) after insertion	1:5	-	+++
	1:7	-	++
	1:10	-	++
Le(a+b-) before insertion	0	++	-
	1:2	++	++
	1:3	++	++
Le(a+b+) after insertion	1:5	++	++++
	1:7	++	++
	1:10	++	++

Table 3. *In vivo* transformation of rat red cells. Le^b-6 glycolipid single doses (over the range of 2.0 – 0.05 mg) dispersed in lipid infusion media were intravenously administered to rats. Red cell samples were taken over 34 days and analysed by serology with anti-Le^b reagent. Results show that cell labelling with glycolipids reached maximal labelling within 1 day, and then after about 1 week were slowly lost (unlike in a natural situation where they would be continuously replenished by the animal and remain at a constant level).

Le^b (mg)	Serology days post in vivo infusion of Le^b glycolipids																	
	1	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30	32	34
2.00	++++	+++	++++	++++	++++	++++	++++	+++	++	++	++	++	++	++	++	+	+	-
1.00	+++	++++	+++	++++	+++	+++	++++	+++	++	++	++	++	++	++	+	-	-	-
0.50	++	++	++	++	+++	+++	+++	++	+	+	+	+	-	-	-	-	-	-
0.25	++	++	++	++	++	++	++	+	-	-	-	-	-	-	-	-	-	-
0.10	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
0.05	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Chapter 2. Glycolipids – First Generation Kode Technology

In order for the author to further elucidate the mechanisms responsible for the Le(a+b+) phenotypes he needed to expand his research skill base to include molecular genetics, enzymes and glycolipid structural analysis. The author was fortunate in 1990 to meet with Professor Rafael Oriol (Figure 5), who invited him to come and work in his lab at INSERM, Paris. Professor Oriol later introduced the author to the glycolipid research team of Professor Bo Samuelsson (Figure 5) in Sweden. After several short-term sabbaticals in both their labs, the author moved to Göteborg University, Sweden (Avdelningen för klinisk kemi och transfusionsmedicin, Glyko-och transplantationbiologi, Sahlgrenska sjukhuset, Göteborgs Universitet) as a post-doctoral research fellow from 1994-1996 (Figure 6) with Professors Bo Samuelsson, Karl-Anders Karlsson and Goran Larsson.

Professor Bo Samulesson
circa 1996

Professor Rafael Oriol
circa 1990

Professor Roy Geddes
circa 2000

Figure 5. The three Professors: Samuelsson, Oriol & Geddes. Bo Samulesson (Göteborg University, Gothenburg Sweden), Rafael Oriol (INSERM, Paris, France) and Roy Geddes (University of Auckland/AUT, New Zealand) were all key enablers and research mentors of the author. The first journal publication on Kode Technology (Frame *et al*., 2007) was dedicated to the memory of Roy Geddes (1940-2006).

Following a chance meeting at a conference in Seattle where the author met his former Dean from the University of Auckland, Professor Roy Geddes (Figure 5), the author returned to NZ in late 1996 to establish a research lab under the stewardship of Roy. From New Zealand the author continued to work collaboratively with the independent research teams of Oriol & Samuelsson on immunochemically characterising, purifying and structurally identifying ABH & Lewis related glycolipids from a variety of different sample materials including red cells, plasma, small and large intestines and meconium (Table 4). Several Swedish post graduate students including Lola Svensson undertook their post graduate glycolipid research in the author's New Zealand laboratories. A summary of the Samuelsson/Oriol glycolipid research collaboration outcomes are as listed in Table 4 (excluding non-glycolipid research such as Candelier *et al.,* 1993c; Larson *et al.,* 1996; Fernandez *et al.,* 1998; Costache *et al.,* 1997; Svensson *et al.,* 2000).

Table 4. Titles in chronological order of publications of author contributions related to **glycolipid research**. Journal published conference/meeting abstracts and patents are not listed but are included in the references.

Reference	Article Title
Henry, 1993a	Further insight into the Lewis histo-blood-group system as revealed from study of Polynesian and Caucasian plasma and erythrocyte glycosphingolipids.
Henry *et al.*, 1993b	Plasma and red cell glycolipid patterns of Le(a+b+) and Le(a+b-) Polynesians as further evidence of the weak secretor gene, Se^{w}.
Henry *et al.*, 1994a	Detection and characterization of Lewis antigens in plasma of Lewis negative individuals. Evidence for extended structure formation as a result of reduced fucosyltransferase competition.
Henry *et al.*, 1994b	Expression of Lewis histo-blood group glycolipids in the plasma of individuals of Le(a+b+) and partial secretor phenotypes.
Henry *et al.*, 1994c	Immunochemical and immunohistological expression of Lewis histo-blood group antigens in small intestine including individuals of the Le(a+b+) and Le(a-b-) nonsecretor phenotypes
Henry *et al.*, 1995b	Structural and immunochemical identification of Le^{b} glycolipids in the plasma of a group O Le(a-b-) secretor.
Henry, 1995c	Expression of Lewis histo-blood group antigens
Henry *et al.*, 1997	Structural and immunochemical identification of Le^{a}, Le^{b}, H type 1 and related glycolipids in small intestinal mucosa of a group O Le(a-b-) nonsecretor.
Ångström *et al.*, 2004	Default biosynthesis pathway for blood group-related glycolipids in human small intestine as defined by structural identification of linear and branched glycosylceramides in a group O Le(a-b-) nonsecretor.
Svensson *et al.*, 2005	Novel glycolipid variations revealed by monoclonal antibody immunochemical analysis of weak ABO subgroups of A.
Svensson *et al.*, 2009	Blood group A_1 and A_2 revisited: an immunochemical analysis
Svensson *et al.*, 2011	The structural basis of blood group A related glycolipids in an A_3 red cell phenotype and a potential explanation to a serological phenomenon.
Svensson *et al.*, 2013	Forssman expression on human erythrocytes: Biochemical and genetic evidence of a new histo-blood group system.

Glycolipid (total non-acid glycosphingolipid) methodology

The process of glycolipid extraction from biological samples is relatively complex and time consuming. The original method was developed at Göteborg University, Sweden by Professor Karlsson (Karlsson, 1987) but with modifications as described in detail in Henry, 1993a. Every step of the process was evaluated by thin later chromatography and anisaldehyde staining.

In brief, the method (Table 5) involves first extracting the total lipid mass from the biological sample by refluxing it with solvents (once with methanol, then twice with a CM2:1 mixture of chloroform(C) / methanol (M) and finally with methanol again). At each reflux solvent change the extract was filtered and saved and the residue transferred back to the extraction flask for the next solvent reflux. All four filtrates were combined were then evaporated dry (with the help of toluene to azeotrope with water from intracellular fluids) in a rotary evaporator. The total lipid mass was then subjected to mild alkaline degradation (3 h with 0.2M potassium hydroxide), made into a 2-phase system by the addition of chloroform and then dialysed for 3-4 days (against running tap water) to separate the alkali stable neutral glycolipids from degraded phospholipids and salts. After recovery of the dialysis bag contents, the dialysed lipids were dried down with a rotary evaporator, again with the help of added toluene and CM2:1 solvent. The next step involved large silica 60 (hand-packed columns) chromatography to remove unwanted cholesterol and fatty acid methyl esters (which were washed

out with large volumes of CM99:1). Absorbed glycolipids were then recovered by elution with CM1:3, followed by 100% methanol and a final wash with CMW40:40:12 (a mixture of chloroform: methanol: water(W)), pooled and dried. The next step was ion-exchange chromatography (on long DEAE-cellulose columns hand-packed in CM2:1) designed to separate the neutral glycolipids and alkali stable phospholipids (mainly sphingomyelin) from the acidic glycolipids (sulfatides/gangliosides). The lipid extract was loaded, and twice per day, over 2 days, was run slowly further into the column (and allowed to equilibrate). After 2-days the column was washed with CM2:1 and methanol to remove the unbound neutral glycolipids and sphingomyelin, which were rotary evaporator dried. If the acidic glycolipids were required they could be eluted with 5% LiCl (for secondary processing) or discarded.

At this stage the unwanted phospholipid sphingomyelin is dominating, so the entire lipid mix was acetylated with acetic anhydride. (Sphingomyelin only has one acetylation site so remains polar, while neutral glycolipids have many acetylation sites and becomes non-polar). The acetylated lipid mix after acetylation was extensively rotary evaporator dried, and then loaded onto a silica-60 column packed in CM98:2. The first fraction was eluted with CM95:5 followed by CM9:1, which eluted the acetylated glycolipids. Sphingomyelin still bound to the column could be eluted with CM1:3 followed by CMW60:35:8.

The acetylated glycolipids now needed restoring back to their native state, and deacetylation was achieved by mild alkaline degradation with potassium hydroxide and dialysis (as described above). After recovery of the dialysis bag contents, the dialysed lipids were dried down with a rotary evaporator. The next step was a second ion-exchange chromatography required to remove alkali-stable and amino group containing phospholipids which had become *N*-acetylated derivatives during the acetylation procedure, thus making them acidic. The same procedure as previous was used to obtain a dry lipid paste.

Finally a last silica chromatography was done to remove any remaining nonpolar contaminants, with the procedure the same as the first silica chromatography (but in much smaller scale). The total non-acid glycosphingolipids obtained by this procedure (about 2.5 mg/100 mL plasma or 4.5 mg/100 mL red cell mass) were now ready for fractionation by HPLC (required for NMR structural analysis), or as is for thin-layer chromatography immunochemical analysis and mass-spectra (Henry *et al.,* 1994a; Henry *et al.,* 1995b; Henry *et al.,* 1997; Ångström *et al.,* 2004), (Figure 6).

Table 5. Outline of Karlsson methodology (Karlsson, 1987) for preparation of total neutral glycolipids

	BIOLOGICAL SAMPLE (100 g)
Step 1.	Solvent reflux extraction of total lipids from biological sample
Step 2.	Mild alkaline degradation and dialysis to remove phospholipids
Step 3.	Silica 60 chromatography to remove cholesterol and fatty acid methyl esters
Step 4.	Ion exchange chromatography to remove acidic glycolipids
Step 5.	Acetylation to facilitate chromatographic separation of glycolipids from sphingomyelin
Step 6.	Chromatographic removal of sphingomyelin and other contaminants from glycolipids
Step 7.	Deacetylation to restore glycolipids back to original state
Step 8.	Ion exchange chromatography to remove acidic contaminants
Step 9.	Silica 60 chromatography to remove non-polar contaminants
	TOTAL NEUTRAL GLYCOSPHINGOLIPIDS (0.002 – 0.005 g)

Figure 6. The author working (at a MS/MS mass spectrometer) as a post-doctoral research fellow. Photo taken at the Avdelningen för klinisk kemi och transfusionsmedicin, Glyko-och transplantationbiologi, Sahlgrenska sjukhuset, Göteborgs Universitet (Göteborg University, Gothenburg Sweden). Photograph *circa* 1995.

It was this experience with glycolipid extraction methodologies, and a background in transfusion medicine, that lead to the author into believing that a commercially viable ABO quality control product could be made with natural blood glycolipids extracted from waste (expired) human blood. The problem was essentially that the methodology of Karlsson (1987) described above, was designed as R&D methodology to prepare a few mg of total glycolipids from a maximum of 2-3 units of blood, and what the author needed was scale methodology able to manage 200-300 units of blood, and be cost efficient. Kiwi ingenuity was needed and to do this, the author returned to New Zealand and established the company Kiwi Ingenuity Limited (see Kode Technology Theme: Kode Biotech Limited).

Large scale natural glycolipid isolation and prototype product

The objective was to extract ABH glycolipids from expired waste blood cells at sufficient purity that they could be inserted into group O cells to make an ABO quality control system. For red cell glycolipid extractions, a series of postgraduate student projects set out to rationalize the methodology (Ristovic, 2001) including a stromal flotation method (Chen, 2001).

The first step was to develop methods for low-cost large-scale preparation of purified blood group glycolipids. This was no small challenge, and required re-thinking of all process steps. Fortunately, the author recalled once seeing a documentary on Columbian cocaine labs operating in the bush and using bathtubs and weed-eaters for their initial processing steps. With these bucket-chemistry concepts in mind, the author developed primary extraction methodology using industrial kitchenware, including large 10L stainless steel cooking pots, food-quality strainers for soup filtration with Chux® Heavy Duty Superwipes as filter membranes, and Baine-Marie's for evaporation drying steps. These methods were very low tech but cost effective and able to reduce 75 kg (about 300 blood bags of expired human blood) of bulk biological material into about a ½ kilo of a lipid paste suitable for further processing. From this paste further processing based on the method of Karlsson (1987) described above, resulted in about 3 g of a total neutral glycolipid mixture, which were predominantly ABO glycolipids, but also contained other glycolipids including Lewis. Further processing was not

economically viable for a commercial product, and so it was this preparation that was to be used to prepare glycolipid modified cells.

The second issue was that these semi-crude total glycolipid preparations were poorly soluble in aqueous buffers (needed for transformation of red cells) and so further development was required. Ultimately it was found that by drying the natural glycolipids dispersed in solvents down onto salts (Gilliver, 2006), they could be rehydrated with water as an aqueous solution suitable for dilution and modification of red cells. The first of these natural glycolipids preparations was formulated as a prototype product to make quality control cells, (that is group O cells modified with inserted levels of ABO blood group glycolipids), and then trialed both in-house and by the Scottish National Blood Service. The key learnings from the earlier natural glycolipid results from the post graduate student work of Ji Chen (Chen, 2001), where the glycolipids were diluted in plasma and later results of Lissa Gilliver (Gilliver, 2006) where PBS was used as a diluent, are summarized in the tables below and are included in the patent "Sensitivity controls for blood serology prepared from modified cells" (Figure 7).

The exemplar results shown in Table 6 for blood group A glycolipids (but with similar albeit slightly less sensitive results obtained for blood group B glycolipids) supporting the use of natural glycolipids to make an ABO quality control product. In other data (not shown) it was learned that the length of the saccharide only had minimal impact on the serological result (Chen, 2001) and intriguingly that transformation with higher HPLC purity (>95%) Le^b glycolipids required about 2 magnitudes less material than a >50% pure mix of glycolipid (Gilliver, 2006), strongly suggesting the need for relatively pure compounds. It was also found that the optimal ratio of mixing packed red cells with natural glycolipids diluted in plasma was about 3:1 (Chen, 2001, see also Table 2) while 1:1 was later found to be a better ratio with PBS diluted glycolipids (Gilliver, 2006). Although the optimal contact time in plasma at 37℃ was 24 hours, almost equivalent results could be obtained at 2-4 h (Table 6), and good results were seen with PBS diluted glycolipids at 2 hours (Gilliver, 2006). The temperature of glycolipid insertion was evaluated, and as expected 37℃ was much more efficient than 2℃ (Gilliver, 2006). Finally, the long-term storage of cells modified with glycolipids and stored at 4℃ was evaluated and it was found that the reactions were stable for about at least 3-4 weeks (Chen, 2001; Gilliver, 2006) (Table 6).

Table 6. Evaluation of stability of the blood group A glycolipid modified red cells over 5 weeks (Chen, 2001). Results show both the effect of glycolipid concentrations on serology and stability.

Time post insertion (weeks)	Serology of glycolipid (mg/mL) modified red cells							
	9.6	7.2	4.8	3.6	2.4	1.9	1.2	0.6
0	++++	++++	++	++	+	-	-	-
1	++++	++++	++	++	+	-	-	-
2	++++	+++	+++	++	++	(+)	-	-
3	++++	+++	+++	++	++	(+)	-	-
4	+++	+++	++	++	+	-	-	-
5	+++	+++	++	++	+	-		
6	++	++	++	+	+	-	-	-

After eight years (1996-2004) of purification development and serological evaluation the natural glycolipid results although not perfect, were considered adequate and able to stably transform group O red cells to have ABH blood group antigens, and be viable as a commercial product. A patent was filed which related to using natural glycolipids and modified glycolipids (Figure 7), and a potential licensee began evaluating their performance. At the same time competitive technologies involving modification of red cells with glycosidases (WO 2004/072306) and glycosyltransferases (WO 2005/121322) were also actively explored, but not commercially pursued, as they had no competitive advantages over FSL modification.

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WO 03/034074 A1

(54) Title: SENSITIVITY CONTROLS FOR BLOOD SEROLOGY PREPARED FROM MODIFIED CELLS

(57) Abstract: The invention provides for a process for preparing a sensitivity control for blood group determination including dissolving an amount of an antigen in water to give an antigen solution of known concentration, contacting the antigen solution with cells to allow insertion of antigen molecules into the cell membranes of the cells to give transformed cells or contacting the antigen solution with cells that have been modified by the insertion of a linker molecule into the membranes of the cells to allow attachment of antigen molecules to the linker molecules to give transformed cells, washing the transformed cells to give a transformed cell solution, and determining the concentration of the transformed cell solution to enable the solution to be used as a sensitivity control for blood group determination.

Figure 7. International patent PCT “Sensitivity controls for blood serology prepared from modified cells”. Inventors (alphabetical order) were Deborah Blake, Lissa Gilliver, Stephen Henry, Jasmine Chen, with a priority date of 16 October, 2001. This patent (WO 03/034074) describes the use of natural glycolipids to make blood group sensitivity controls.

Biotinylated gangliosides

From 1999 through to the end of 2005 biotinylated natural ganglioside glycolipids (using avidin as a bridge) were used to attach a variety of biotinylated glycan and proteins onto cells (Blake, 2003; Carter, 2007; Gilliver, 2006; Patel, 2008) and embryos (Blake, 2003; WO 2003/087346). Gangliosides were biotinylated using a modified method based of Wilchek & Bayer (1987). In brief, gangliosides (a side product from the preparation of the neutral glycolipids - Table 5) were first periodate oxidised with sodium m-periodate, then biotinylated with biotin amido propyl hydrazide BACH, and reduced with potassium borohydride, with dialysis between stages and final drying. The resultant product prepared was termed as BioG (for biotinylated ganglioside) and could be solvent-assisted to disperse into PBS.
It was established that BioG modification of red cells could be achieved with incubation for 60-90 minutes, resulting in BioG cells (Blake 2003). A proof-of-concept experiment with biotinylated blood group A glycans (on a polyacrylamide backbone) produced strong serological results (Blake, 2003). Additionally biotinylated antibody (anti-A,B) and lectin (*Ulex europaeus*) were attached to BioG-red cells and BioG-embryos (via an avidin bridge) and were successfully able to react as anticipated and cause rosetting of antigen-positive cells on the surface of the BioG-avidin-biotinylated IgG/lectin modified cells (Blake, 2003).

Extension serologic research with BioG red cells and biotinylated glycans established that the range for BioG to modify red cells was between 50-500 µg/mL in order to achieve acceptable serology (Gilliver, 2006). Although the method worked in principle, there remained several significant issues, primarily related to sensitivity for blood group B and storage stability, and so this approach was abandoned as the newly arrived synthetic glycolipids (see below) were showing promise. However, proof-of-concepts were established and the need for a biotinylated synthetic construct identified. In October 2003 a request for such a synthetic biotin construct was made, as seen in the (unrelated content redacted) email conversation below.

From: bovin@carb.ibch.ru
To: "Steve Henry" <stephen.henry@aut.ac.nz>
Date: 1/10/2003 1:23:50 a.m.
Subject: Re: KIL4801 E15

Dear Steve,
Glad to learn about good results with Atri-DOPE
[redacted]
As for biotinylated DOPE, it is possible to produce it, but it will surely be completely insoluble in water. It is possible to prepare GLYCOlipid with biot, but it is difficult. However, its physical properties will be different from those of glycolipids, in particular with respect to insertion to cells. I think that this will be a time loss, only radiolabel will help in this case

With best wishes
Nicolai Bovin

Although this request was unfulfilled at the time, it stayed on the agenda until 2007 when the invention of the CMG spacer, allowed Nicolai and his team in 2008 to successfully made a synthetic biotinylated construct (termed FSL-biotin, see Chapter 4: CMG Spacers – Third Generation Kode Technology and Kode Technology Theme: Fluorophores & biotin). The FSL-biotin construct would become one of the most powerful research products in the Kode Technology portfolio.

Chapter 3. Synthetic Glycolipids – Second Generation Kode Technology

Although the results from the natural glycolipids to prepare antigen modified blood cells for quality control use were adequate, the methodology of extracting glycolipids from red cells was only marginally commercially viable, and dilution in plasma was not ideal. Ideally what was needed was glycolipids of much higher purity, an ability to have standardization between batches, to be much more cost effective, and not to cause any harm to the modified cell. The only feasible approach which seemed possible was to try and make synthetic glycolipids. In July 1997 exploratory discussions on potential synthetic glycolipids (specifically Lewis structures) were initiated with Nicolai V Bovin (Figure 8), a renowned glycobiologist and synthetic chemist (and colleague who the author had met a decade earlier during one of his sabbatical research visits with Professor Rafael Oriol). In that email (14 Jul 1997) Nicolai states (where OS means oligosaccharide)

> "*we will convert OS to OS-NH2, then to OS-NHCOCH2NH2 followed by acylation of amino group with a lipid acid, e.g. palmitic one or other (by your choice) … (b) it is possible to conjugate OS with a lipid containing two lipid residues (as in ceramide) but the work will be considerable higher."*

Despite this potential concept being considered in 1997 nothing progressed further until 2002.

Nicolai V Bovin
circa 1995

Figure 8. Professor Nicolai V Bovin, glycobiologist, synthetic chemist and co-founder of Kode Technology. The author and Nicolai have been colleagues since 1990

The birth of the synthetic FSL construct and Kode Technology

It was not until late-2002/early-2003 that the synthetic glycolipids route was actively pursued and evaluated. Due to the specialist skills required in serology, the author (a former immunohematology- red cell serologist by training) read and scored all serologic reactions. The author asked Nicolai if could he make some synthetic ABO glycolipids, which Nicolai had at this time been experimenting with. The first two prototype synthetic constructs were received in February/March 2003. Experimental lab notes were lost but email correspondence (reproduced verbatim) that identified they didn't work was as follows.

From: Steve Henry
To: bovin <bovin@carb.siobc.ras.ru>
Date: 14/03/2003 8:25:27 a.m.
Subject: Kiwi KIL3003 E20

Hi Nicolai
We have extensively tested the samples that you sent us at a range on concentrations including undiluted. We were not able to get any serological results from the molecules sent, ie they did not insert into the red cell membrane or were not detectable. Without any knowledge of their structure I would suspect that either they did not have a bi-lipid tail or the bi-lipid tail was too short. Alternatively they may have been too short to be seen by antibodies when in the membrane. Could you please describe the basic structure of the molecules sent. We are going into field trials with the natural molecules very soon, and although we are not dependent on the synthetic molecules we would like that sometime in the near future the natural product could be replaced by a synthetic molecule made by you. The market size is quite significant.

>>>bovin <bovin@carb.ibch.ru> 03/15/03. 00:33 AM >>>
Dear Steve,
Thank you for your message. One of the derivatives contained a short spacer, the second one had oligoethyleneglycol spacer, about 40 angstrom. It seems that the spacer must be even longer. In addition, we can try a very large PAA molecule bearing several PE residues and a lot of trisaccharide residues.
So, let us try more…

With best wishes
Nicolai Bovin

In August 2003 the authors team received the second batch of three prototype synthetic glycolipid constructs (prototypes 3-5). (Experimental lab notes and email correspondence (including an appended technical report-not shown) were used to reconstruct the history).

The third candidate had a blood group A trisaccharide, a short PEG (*n* =10) spacer and dioleoylglycerol lipid (A_{tri}-sp-lipid). This construct was unable to go into solution phase in aqueous buffer despite extensive heating, sonication, and solvent-assistance. All the same the resultant cloudy solution (in PBS) was contacted with red cells, but it caused no transformation of the red cells (Figure 9).

The fourth candidate had a B trisaccharide with adipate spacer and a dioleoylphosphatidylethanolamine lipid tail) and was called (B_{tri}-sp-Ad-DOPE). This construct rapidly and easily dispersed as a clear solution in PBS and was able to modify red cells (Figure 9).

The fifth candidate had a B trisaccharide with a polyacrylamide spacer (2,000,000 Mw) and a palmitoylethanolamide lipid (B_{tri}-PAA-PEA). This construct was also poorly soluble in aqueous buffers despite extensive heating, sonication, and solvent-assistance. All the same the resultant clear solution in PBS but containing insoluble crystal residue, was contacted with red cells, but again caused no transformation of the red cells (Figure 9).

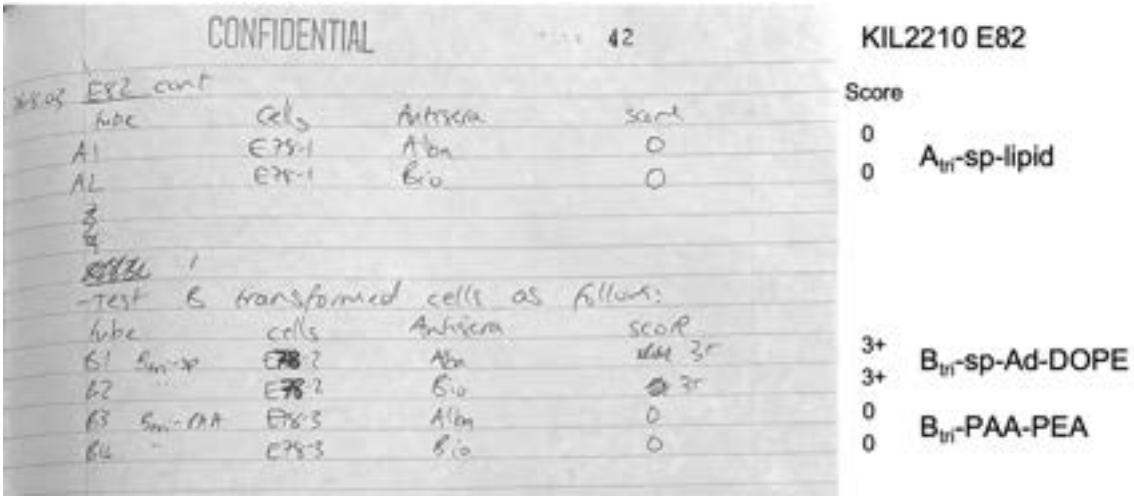

Figure 9. Experimental lab notes showing the first positive serological results for a synthetic glycolipid. Notes from the Kiwi Ingenuity Limited (Lissa Gilliver), laboratory notebook KIL2210 E82 on 26th August 2003. Serology reactions were observed and graded by the author and the experiment was set-up and recorded by Lissa. To the right of the image has been added a typescript annotation to aid with interpretation of the key concepts in the hand-written lab book.

Although in 2003 the term Kode Technology had been adopted for the natural glycolipids (see Preface) they were actually just the first concepts, and discovery of the synthetic glycolipids (26th August 2003) which displaced them, are now considered the birth of Kode Technology. Also at this time modified cells were not yet known as kodecytes (although they were called KODE cells in 2005!) the term kodecyte would not appear until May 2009, see Preface). Synthetic constructs were also called SYN for synthetics from 2003 (Frame *et al.*, 2007) but became known as Function-Spacer-Lipids (FSL) in 2009 (Henry, 2009).

Immediately following these 2003 preliminary results the author informed Nicolai Bovin and requested A_{tri}-sp-Ad-DOPE for evaluation. Email correspondence (reproduced verbatim, but with commercially sensitive redactions in grey) between the author and Nicolai Bovin relating to the success of the B_{tri}-sp-Ad-DOPE and the request to make an A_{tri} equivalent. The final sentence of the author's first email paragraph 1 " … then they will be used to replace the natural material" forebodes the end for the natural glycolipids, while the last email indicates a high level of confidence a product would result.

From: Steve Henry
To: bovin
Date: 26/08/2003 2:37:34 p.m.
Subject: Kiwi

Hi Nicolai
Some very good news. We have been testing the reagents you sent and have found one that works very well. We will send a full report at the end of this week regarding all the other reagents. In the meantime the product Btri-Sp-Ad-DOPE (E672) gives excellent results. We will now take this product into stability trials. Could you please make some Atri-So-Ad-DOPE as soon as possible and send it to us. If these product both have adequate stability we will put them into the field trial and if they survive then they will be used to replace the natural material.
We would imagine that the annual usage of the product if it survives the stability trials will be around 10-100 grams per year for each A and B. Is this viable from your end? Could you also indicate an initial price for say 1 gram of each.

>>> bovin@carb.ibch.ru 26/08/2003. 10:50:07 p.m. >>>
Dear Steve,
Indeed, very good news! We will send you Atri-sp-DOPE asap. If the stability of these substances proves to be insufficient, we will try to find a way to increase it. It is possible to produce 10-100g annually, if this is done gradually, say 5 g in the first year, 20 in the second, 100 g in the third. I can give only the most approximate estimates concerning the price, naturally, it will also be depending on production scale. By now the supposed interval is $[redacted] per 1g.
With best wishes,
Nicolai Bovin

From: Steve Henry
To: bovin
Date: 29/08/2003 2:54:47 p.m.
Subject: Kiwi KIL4801 E10

Hi Nicolai
Our report on the reactivity of the reagents sent is attached.
we are all very excited about the results so far and a very confident it will result in a product.
We will need to jointly protect the intellectual property but we can discuss this when we prove the stability is OK and the blood group A is OK.
Could you please advise of a time frame for the A molecules.
If the A works OK then within a month we will need another sample of both A and B to undertake the field trials. If they work then we can start selling the product and you will need to make perhaps a gram of each within a month or so. We will keep you fully informed of the time frames because as soon as we launch you will need to be able to supply the product to meet the demand – which I predict will grow very fast to at least 100 grams of each per annum.
Very best wishes
Steve

On 23 November 2003 the sixth prototype being FSL-A trisaccharide (A_{tri}-sp-Ad-DOPE) with the same architecture as B_{tri}-sp-Ad-DOPE, arrived and it performed exactly as anticipated in its ability to appropriately modify red cells (results not shown). Both constructs performed exceptionally well and gave excellent serological results. Over the next few months a variety of different synthetic glycolipid spacer and lipid variations were evaluated (Gilliver, 2006) for their water solubility and ability to modify cells (Table 7) including those listed variations listed in Table 8. Of note was that monoacyl lipids did not modify cells.

Synthetic name	Abbr	Water solubility	Detectable transformation
A_{tri}-sp-Ad-DOPE	Syn A	Yes	Yes
B_{tri}-sp-Ad-DOPE	Syn B	Yes	Yes
A_{tri}-spisp2-Ad-DOPE	A-sp_2	Yes	Yes
A_{tri}-sp-Ad-DSPE	A-DSPE	Yes	Yes
Lipophilic A_{tri}		Yes	No
A_{tri}-sp-POE_{10}-DOG	A-DOG	No	No
B_{tri}-PAA-DOPE	B-DOPE	No	No

Table 7. The ability of seven different ABO blood group synthetic glycolipids variations to disperse in water/PBS and transform cells (Gilliver, 2006).

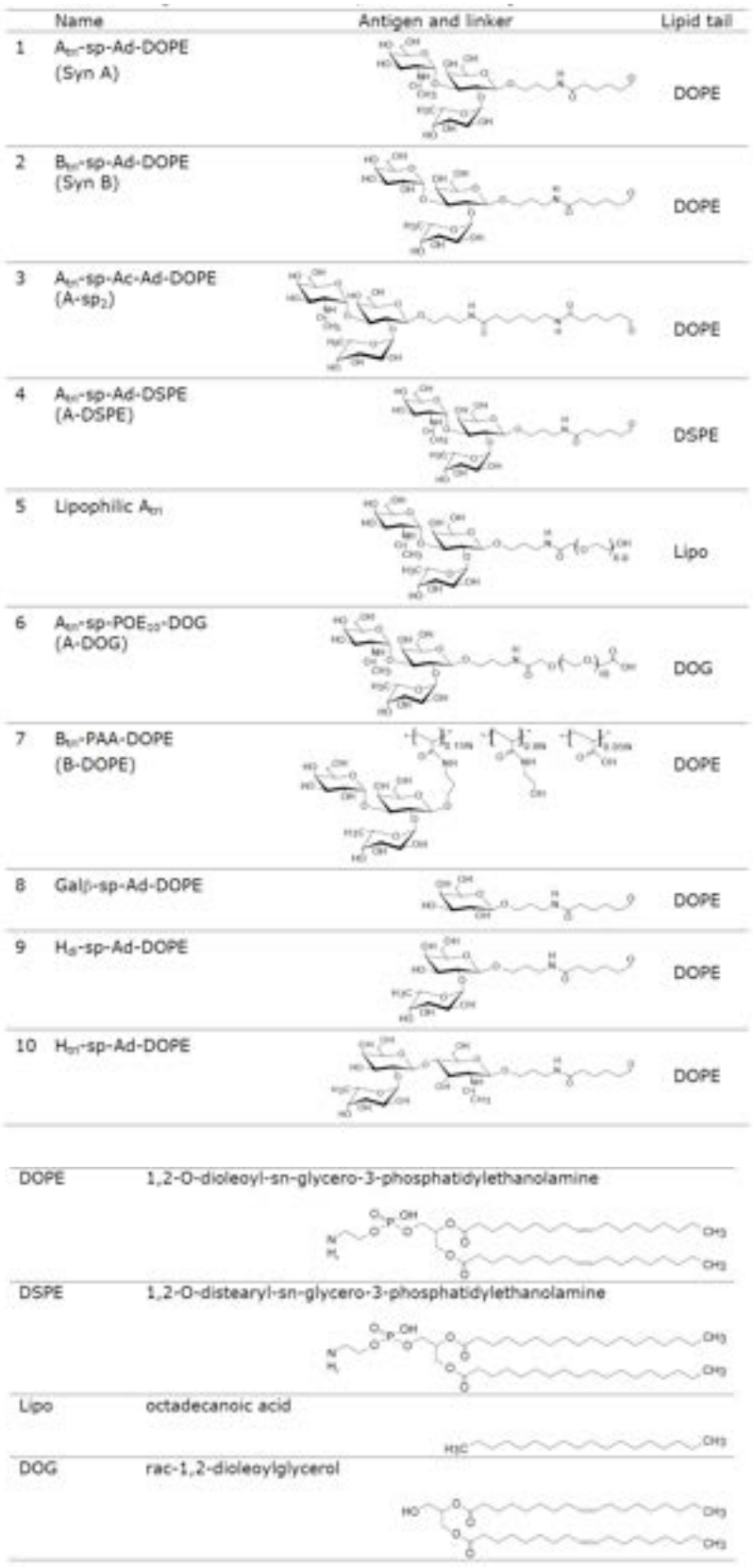

Table 8. Variations in synthetic ABH and related blood group synthetic glycolipids tested. Original figure from (Gilliver, 2006) including structures of the four lipid moieties.
Structures 1-7 relate to Table 7.

The original method for modification of cells (derived from natural glycolipids, was to disperse the constructs in 70℃ PBS then use a ratio of 3:1 packed red cells to transformation fluid and incubate for 1 hour at 37℃, wash then store in a preservative solution). However in 2010 the methodology (following extensive evaluations, and refinements for large scale manufacture) changed to dispersing the constructs at room temperature in PBS (or cell preservative solution), mixing and then contacting 1 part of FSL construct solution with 1 part of packed cells for 1 hour at 37℃.

Patent and water (aqueous media) dispersible feature

The paradigm of the time (including our own natural glycolipid experiences was that glycolipids required solvents/lipids to assist them into solution phase. However the glycan-adipate-DOPE synthetic glycolipids easily dispersed in PBS. This was an unanticipated outcome and a major new finding. The ability to have PBS dispersible (in the absence of any solvents) constructs that would spontaneously and harmlessly insert into cells was a significant step-change, and became the guiding principle in the design of all future Kode Technology constructs, and a key novel feature in the subsequent patent filings (Figure. 10)

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(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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WO 2005/090368 A1

(54) Title: SYNTHETIC MEMBRANE ANCHORS

(57) Abstract: The invention relates to synthetic molecules that spontaneously and stably incorporate into lipid bi-layers, including cell membranes. Particularly, although not exclusively, the invention relates to the use of these molecules as synthetic membrane anchors or synthetic molecule constructs to effect qualitative and quantitative changes in the expression of cell surface antigens.

Figure 10. Patent filing “Synthetic membrane anchors” (WO 2005/090368) claiming the new synthetic glycolipids

The four key claims (of which there are 125) in this patent (WO 2005/090368) are claims 14-17 as follows:

(14) A **synthetic molecule construct** of the structure F-S_1-S_2-L where:

- F is an antigen selected from the group consisting of carbohydrates, proteins, lipids, lectins, avidins and biotin;
- S1-S2 is a spacer linking F to L; and
- L is a lipid selected from the group consisting of diacyl- and dialkyl-glycerolipids, including glycerophospholipids, sphingosine derived diacyl- and dialkyl-lipids, including ceramide.

(15) The synthetic molecule construct according to claim 14 where the synthetic molecule construct is water soluble.

(16) The synthetic molecule construct according to claim 14 or 15 where the synthetic molecule construct spontaneously incorporates into a lipid bi-layer when a solution of the synthetic molecule construct is contacted with the lipid bi-layer.

(17) The synthetic molecule construct according to claim 16 where the synthetic molecule construct stably incorporates into a lipid bilayer.

The first commercial product arises

Immediately after the discovery of the synthetic glycolipids, although work was still running with evaluating purified natural glycolipids (and was not abandoned until early 2005), simultaneous work was started on evaluating the synthetic glycolipids. The next stage-gate in product development was to establish if the synthetic glycolipid construct modified cells would be stable as a reagent cell commercial product (which typically have a shelf life of up to 6-8 weeks). Samples of both synthetic constructs were set up for long term stability trials and also sent to CSL (Australia) for independent evaluation. After multiple and extensive independent evaluations, both teams found that modified cells were stable, and showed no loss in activity after 2 months of storage in cell preservative media.

The final stage-gate of evaluation was extensive field trials. One part of this organised by CSL Biosciences (Tim Carrol, Production and Development, CSL) was for the modified cells to be included in a Royal College of Pathologists Australasia (RCPA) Educational Survey Exercise scheduled for 22nd March 2004. In conjunction with Kode Biotech, CSL manufactured the 4 samples for the RCPA Educational exercise, and the RCPA distributed the survey to 310 Australasian laboratories, who undertook blind evaluation.
The trial was a huge success (and was repeated by the RCPA in 2008) and identified some deficiencies in blood group quality control. The outcomes from both trials were published by the author (Henry, 2009). On the basis of this outcome CSL Biosciences started the development of a ABO sensitivity control commercial product called Securacell™, which was released to the market on February 10th 2005, and is still being sold in 2023 (See Kode Technology Theme: Glycans, Figure 13), albeit it now with third generation Kode constructs.

Chapter 4. CMG spacers – Third Generation Kode Technology

Although the initial applications for Kode constructs was as synthetic glycolipids (primarily for research and making the ABO sensitivity controls) it can be seen in the 2001 business plan overview (see Kode Technology Theme: Kode Biotech Limited), two years before the synthetic glycolipids existed, that there were identified commercial drivers for peptide based products. Furthermore, with the success of the CSL synthetic glycolipid Securacell® ABO sensitivity control product in 2005, interest from CSL (Tim Carroll/Damien Heathcote) for a variant MNS (Miltenberger, vMNS) FSL-peptide construct was strong (see Kode Technology Theme: Peptides & Proteins, Figure 18 for details).

In November 2004 three potential Miltenberger peptide-DOPE (no-spacer) constructs were received for evaluation (Figure 11). The issue with these new constructs was their insolubility in aqueous media and so significant effort was put in to find ways to facilitate their dispersion in media so that they could be used to label cells. Over the next several weeks, attempts at dispersing the constructs in biologically compatible media included use of mild detergents, added lipids, heat, sonication, acidification, solvents and combinations thereof. However, when analysed for ability to modify red cells the results were always hemolysis and/or cell denaturation and as there was never any evidence of modification or bioactivity this approach was abandoned. It was clear that the guiding principle requirement for Kode constructs to be dispersible in PBS without any solvents, and spontaneously and harmlessly insert into cells was going to be a challenge.

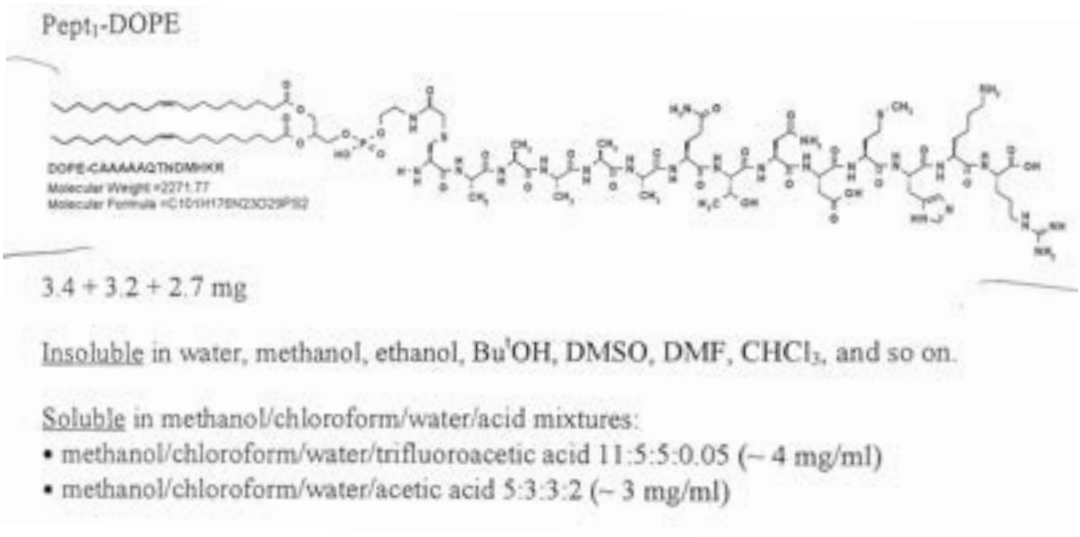


Figure 11. Photograph of lab-book note (10/11/04) of the first peptide-lipid constructs received. It was clear from the description provided that solubility in biologically compatible aqueous media was going to be a major issue.

Polyethylene glycol (PEG) & ethylene glycol (EG)

In March 2005 the second attempt arrived in the lab, being a peptide-PEG-DOPE construct. This construct dispersed well in PBS and appeared able to modify red cells. However, it was interesting to note that the modification also appeared to block intrinsic antigens on the red cells, as noted in the following (redacted) author email to Nicolai (see below). These unexpected results raised the possibility to use PEG constructs

as masking agents and in August 2005 two new PEG constructs arrived in our hands being PEG_{2000} and PEG_{5000} (where the number relates to the MW of PEG, and their contour length being 13 & 32 nm, respectively) to evaluate this. Although these constructs found no value with red cell serology antigen masking, in 2013 FSL-PEG constructs were successfully used to mask antigens on viruses (Tesfay *et al.*, 2013) (see Kode Technology Theme: Virology and infectious diseases).

From: Stephen Henry
To: bovin <bovin@carb.siobc.ras.ru>
Date: 24/03/2005 9:41:36 a.m.
Subject: Kiwi

Hi Nicolai

We have trialled the Miltenberger PEG-DOPE molecule and find that is seems to insert however, the PEG appears to mask the other antigens on the cells. You may recall that attaching large amounts of PEG to cells can actually mask antigenic structures. This appears to be what has happened and although not useful for attaching peptides it potentially is a very valuable result (we will pursue this separately). Do you have enough peptide to manufacture a new series of molecules if not please advise and we will order more. This time the linker needs to be very much smaller – say 3-5, or perhaps a maximum of 10 PEG molecules. Can this be done?

best wishes
Steve

>>> Nicolai Bovin <bovin@carbohydrate.ru> 25/03/2005 9:44:49 p.m. >>>
Dear Steve,

We will synthesize a derivative with about 10 EG units. I think that lesser number will lead again to an insoluble product.

With best wishes,
Nicolai Bovin

The initial Miltenberger-PEG-DOPE (2005) construct and the PEG_{2000}-DOPE constructs were further evaluated in a variety of serological platforms. Despite some promise, their serologic characteristics in diagnostic platforms was somewhat atypical (sticky) and a relatively large proportion of the population reacted apparently non-specifically, with what was later identified to be PEG antibodies. Along with the masking issues identified above, the use of PEG as a linker/spacer was abandoned. But not entirely, and instead it was changed to a 1.8 nm short version in the form of ethylene glycol (EG_6, with 6 glycol units). Also at this stage the new guiding principle for a Kode construct became expanded to “the construct must be PBS dispersible in the absence of any solvents, AND spontaneously and harmlessly insert into cells AND must not non-specifically react with serum”.

In April 2006 two new peptide-EG_6-DOPE Miltenberger constructs were developed. Over the next year these were soon followed by seven new peptide-EG_6-DOPE constructs, and all were put into extensive serological evaluation. However during this period, and although the EG_6-spacer construct seemed to have promise (WO 2009/035347), Nicolai and his team were investigating an alternative approach; a new spacer, called carboxymethyl glycine (CMG).

In early 2007 a syphilis peptide-PEG_{14}-DOPE construct was also developed but showed very poor correlation with EIA validated samples, and 26% of negative samples reacting positive due to the PEG spacer antibodies (see Kode Technology Theme: Peptides & Proteins, Figure 19) (Patel, 2008).

Carboxymethyl glycine (CMG)

Despite being able to make small-scale versions of carboxymethyl glycine (CMG) spacers as early as 2006, scaling-up was difficult, and it took until August 2007 before the first peptide-CMG-DOPE constructs arrived in the author's lab for testing. Unexpectedly this was to be a watershed event for Kode Technology, as the new CMG spacer facilitated further opportunities for the Kode Technology portfolio (WO 2009/048343), and also enabled the long awaited biotin construct to be made (arriving in April 2008) (see Kode Technology Theme: Fluorophores & biotin). For an extensive review of the structure and characteristics of CMG, the "Goldilocks" spacer, see the Kode Technology Illustrated Technical Manual (Appendix 2; *Henry et al, 2023*).

With the invention of the CMG spacer the peptide constructs became the new focus and the primary project was the development of a Miltenberger diagnostic. Over the next 15 months (April 2007- July 2008) 21 Miltenberger constructs were developed and evaluated by the author's lab and CSL. In 2009 this development became the second license product AbtechCell™ III and Phenocell™ C, and like Securacell™ is still being sold today (See Kode Technology Theme: Peptides & Proteins for further details).
The other peptide construct at the time was a syphilis diagnostic (2007-2010), which although the prototype worked well (Komarraju *et al*., 2010; Chesla *et al*., 2010) was unable to find a commercial niche and so was shelved. In 2019 the syphilis project was resurrected as a development precursor for the design of a Leptospira diagnostic (see Kode Technology Theme: Peptides & Proteins), which also resulted in improved sensitivity and specificity for the syphilis assay (Nagappan, 2022).

In 2023 while writing this DSc thesis the author asked Nicolai what was the background to CMG. The following quote is Nicolai's recollection of events leading to the development of the CMG spacer

> *"This is the story:*
> *When planning the synthesis of conjugated hydrophobic peptides, we realised that they would produce FSLs insoluble in an aqueous medium, so it is necessary either to add several hydrophilic amino acids to the peptide chain (which is fraught with an unpredictable change in properties), or to tether L and the peptide with a polyethylene glycol spacer. In practice, the latter did indeed dissolve well, but to our surprise, approximately 15% of the peptide-negative sera bound to them. Subsequently, we also found data in the literature on a rather frequent interaction of human antibodies with PEG. In an effort to avoid the PEG antibody problem we initially constructed an ethylene glycol spacer version for evaluation. However we felt at a dead end, and that it was necessary to come up with a non-trivial hydrophilic spacer. After about 30 minutes brainstorming and working with the requirements: the spacer must have several negative charges, be easy to synthesize and absolutely non-antigenic, the first idea was an oligoglycine chain into which residues of glutamic or aspartic acid are inserted, but this was rejected. Next was proposed to provide the oligoglycine chain with pendant carboxyl groups attached at nitrogen atoms, which led, firstly, to a completely achiral molecule, and secondly, it promised the absence of antigenicity. Almost without discussion, it was clear that there would be one N-carboxymethyl residue per two glycine ones, and there would be six such CMG repeats in the spacer. The chemical synthesis sketched on the blackboard seemed simple (holy naivete!) and we started the synthesis the next day. Indeed, the synthesis of a 20-mg amount proved to be simple, resulting in three FSLs, the first with a Miltenberger peptide followed closely by a syphilis peptide and a year later by one with a biotin residue, which to our common satisfaction, showed the expected nice properties. However, at the next step, an "ambush" awaited us. The 10x scaling itself did not cause any difficulties at the synthesis stage, but the isolation turned out to be extremely painful, due to the need to use chromatography of large volumes of a high viscosity solution, the purification lasted for weeks, and we did not even want to think about further scaling. So we suffered (several chemists, in turn) for several years, until Alexander Tuzikov came up with an elegant alternative synthesis. But that is another story."*

Chapter 5. Kode Technology Application Themes

This chapter is organised into five themes. The first four themes are on glycans, peptides & proteins, fluorophores & biotin, and "other" Kode Technology constructs. Each of these themes discusses the *authors* published contributions to Kode Technology, and mentions some research of independent researchers. A more comprehensive overview on Kode Technology constructs and applications, can be found in the Kode Technology Illustrated Technology Manual (Appendix 2, *Henry et al., 2023*), which also aligns independent Kode Technology publications. The fifth theme discusses the role of the commercial entity Kode Biotech Limited, led by the author (Founder, CEO & Managing Director), as the development of Kode Technology would probably not have happened without the entity Kode Biotech, a commercial vehicle solely focussed on Kode Technology.

It is of note that the different themes (and content within themes) are not necessarily in chronology order, as multiple themes were being simultaneously developed. Furthermore there will be an overlap of some journal articles listed in different themes, as some journal articles are represented by more than one theme.

The pre and early history of Kode Technology development and the author's involvement up to about 2008 has been presented in the preceding chapters, where essentially the foundations of Kode technology was established. Post-2008 there were still a few more important events to happen (Figure 12), but each event was more an incremental improvement on the technology (e.g. variations of CMG) or new application.

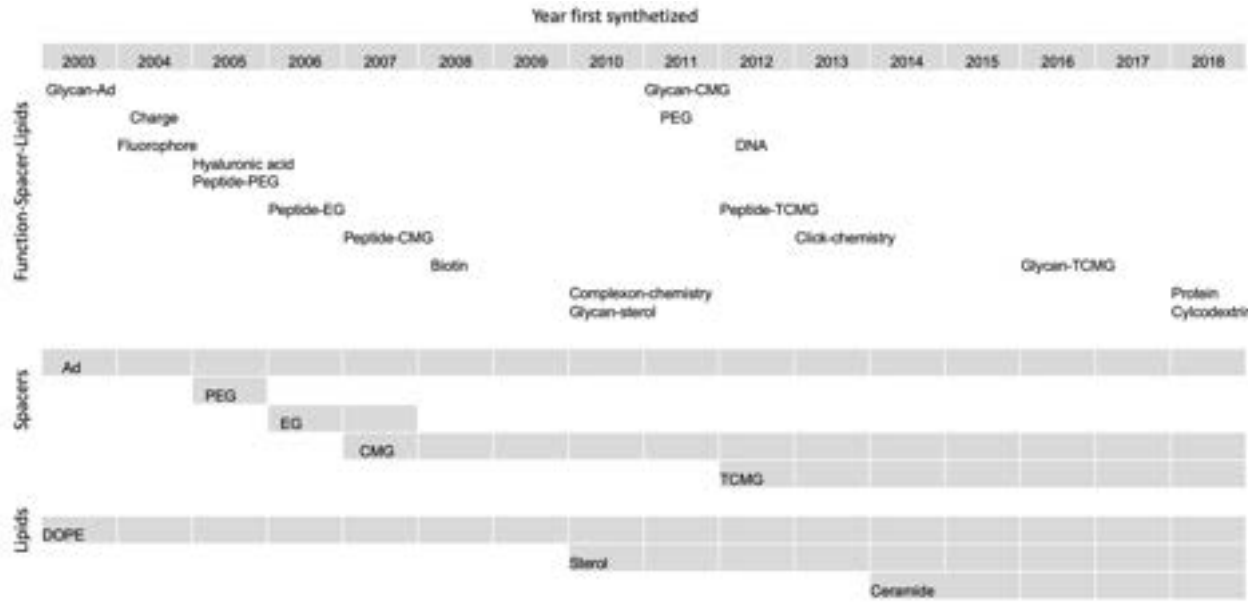


Figure 12. A simplified timeline of key events from the invention of the first successful synthetic Kode technology construct in 2003 until 2018. Shown are the time points when the first example of a family of constructs was developed, or when a new type of spacer or lipid was used. See Kode Technology Illustrated Technology Manual (Appendix 2, *Henry et al., 2023*) for details.

As mentioned earlier, although Kode Technology originally started with glycolipids, it was when the first synthetic glycolipids were developed in 2003 and demonstrated their superior performance over natural glycolipids that the term Kode technology became synonymous with synthetic Function-Spacer-Lipid constructs (and natural glycolipids were relegated to the past).

From the first Kode constructs developed in August 2003 and until the end of 2022 a total of 302 different FSL constructs have been successfully made (Table 9). Successfully made refers to the fact that the constructs met the parameters of "the construct must be PBS dispersible in the absence of any solvents, AND spontaneously and harmlessly insert into cells AND must not non-specifically react with serum", but it does not necessarily mean the construct was suitable for the intended application. Typically for development of a peptide Kode diagnostic assay, using a well developed in-house selection algorithm (Nagappan *et al.*, 2021) about 5-8 different FSL constructs are initially built, from which the most promising 3-4 are selected for intensive biological analysis, and from which 1-2 constructs with the best specificity, avidity and affinity are finally selected. Usually a further round of 2-3 constructs are made, as subtle variations on the best first round constructs, and then the best of the best are selected for product development.

Glycan Kode diagnostics are generally easier to design as glycans are more discrete and development of FSL glycan constructs typically only requires determination of the size of the glycotopes required, and acceptable degree of unavoidable cross-reactivity, both of which substantially influence bioactivity (Barr *et al.*, 2014). However, in contrast to peptides, synthesis of glycans is much more complex.

Number of FSL construct variations synthesized	
Peptides	125
Glycans	82
Hyaluronic acid	16
Other	15
Charge	12
Complexon-chemistry	12
Fluorophore	10
Cyclodextrins	10
Oligo	8
Biotin	6
Click-chemistry	4
Proteins	2
Total	302

Table 9. Number of different Kode constructs successfully synthesized in each family category between 2003-2022. This list does not include the numerous failures, or concepts which remained on the drawing board, or novel constructs in current development.

Although by count the number of Kode peptide constructs made exceeds the number glycan constructs made (Table 9), that number somewhat distorts actual areas of effort and focus. The number of journal publications including published abstracts (associated with the author) have a count of 39 articles primarily based on glycans compared with 19 articles primarily based on peptides, which is a better measure of effort and focus.

In the following themes journal publications (associated with the author) are linked to a copy of the publication via Appendix 1: Published Kode Technology Journal Papers. However, there are three other forms of publications related to the author's contribution to Kode Technology, being conference/meeting presentations and posters, patent applications, and post graduate supervision (resulting in a thesis). The titles of conference/meeting presentations and posters where the abstract has been published in a journal are listed in the references only. Patent applications (drafted by the Kode Biotech patent attorney Dr Stephen Parker) are in listed in the references under their WO publication number (hyperlinked to the WIPO data base) and where the author is an inventor are indicated in blue font. Postgraduate primary supervisions (resulting in a thesis) are listed in the references, with the student name in blue font.

Kode Technology Theme: Glycans

A total of 28 journal papers (Table 10), 11 published conference abstracts and 12 patent applications on Kode Technology associated with the author and related to glycans have been published. This section is divided into 8 sub-themes being:

- Serologic quality control and training
- Mapping antibodies specificities with kodecytes and koded surfaces
- Antibody quantitation
- Hemolysis
- A potential immuno-oncotherapeutic
- Modelling transfusion reactions and antibody neutralization
- Microbiological applications
- Hyaluronic acid oligomers

Table 10. Journal publications titles of author contributions related to Kode Technology Theme: Glycan related papers. Journal published conference/meeting abstracts and patents are not listed but are included in the references. Access to copies of these articles is via Appendix 1

Reference	Article Title
Frame *et al.*, 2007	Synthetic glycolipid modification of red blood cell membranes
Henry, 2009	Modification of red blood cells for laboratory quality control use
Harrison *et al.*, 2010	A synthetic globotriaosylceramide analogue inhibits HIV-1 infection in vitro by two mechanisms
Oliver *et al.*, 2011a	Modeling transfusion reactions and predicting in vivo cell survival with kodecytes
Oliver *et al.*, *2011b*	In vivo neutralization of anti-A and successful transfusion of A antigen incompatible red cells in an animal model
Georgakopoulos *et al.*, 2012	An improved Fc function assay utilising CMV antigen coated red blood cells generated with synthetic Function-Spacer-Lipid constructs
Blake *et al.*, 2011	A simple method for modifying cell/virion surfaces with a range of biological markers without affecting their viability
Harrison *et al.*, 2011	A novel VSV/HIV pseudotype approach for the study of HIV microbicides without requirement for level 3 biocontainment
Hult *et al.*, 2012	Flow cytometry evaluation of red blood cells mimicking naturally-occurring ABO subgroups following modification with variable amounts of FSL-A and B constructs
Korchagina *et al.*, 2012	Toward creating cell membrane glycolandscapes with glycan lipid constructs
Henry *et al.*, 2012	Modeling transfusion reactions with kodecytes and enabling ABO-incompatible transfusion with Function-Spacer-Lipid constructs
Barr *et al.*, 2014	Mapping the fine specificity of ABO monoclonal reagents with A and B type-specific FSL constructs in kodecytes and inkjet printed on paper
Barr *et al.*, 2015	Monoclonal anti-A activity against the FORS1 (Forssman) antigen
Korchagina & Henry, 2015	Synthetic Glycolipid-like Constructs as Tools for Glycobiology Research, Diagnostics and as Potential Therapeutics
Perry & Henry, 2015	Training students in serologic reaction grading increased perceptions of self-efficacy and ability to recognise serologic reactions but decreased grading accuracy
Barr *et al.*, 2016	Biofunctionalizing nanofibres with carbohydrate blood group antigens
Williams *et al.*, 2016a	Glycomapping the fine specificity of monoclonal and polyclonal Lewis antibodies with type-specific kodecytes and FSL constructs on paper
Williams *et al.*, 2016b	Ultra-fast glyco-coating of non-biological surfaces
Perry *et al.*, 2016	Antibody complement-mediated hemolytic studies with kodecytes reveal human complement utilized in the classical pathway is more stable than generally accepted
Ryzhov *et al.*, 2016	Function-Spacer-Lipid constructs of Lewis and chimeric Lewis/ABH glycans. Synthesis and use in serological studies
Henry *et al.*, 2018	Applications for kodecytes in immunohematology
Henry & Bovin, 2018	Kode technology – a universal cell surface glycan modification technology
Perry *et al.*, 2019	A standardized kodecyte method to quantify ABO antibodies in undiluted plasma of patients before ABO incompatible kidney transplantation
Popova *et al.*, 2019	Synthesis of blood group Forssman pentasaccharide GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ–R
Henry, 2020	Kodecytes: Modifying the surface of red blood cells
Perry *et al.*, 2020	Incidence in plasma of low level antibodies against three xenotransplantation and immunotherapeutic glycan antigens
Holmberg *et al.*, 2022	National Blood Foundation 2021 Research and Development Summit: Discovery, innovation, and challenges in advancing blood and biotherapies
Slivka *et al.*, 2022	Influence of the lipid moiety structure on the insertion/release of glycolipids in/from the cell: A study with synthetic analogs

Serologic quality control and training

The first Kode Technology journal publication "Synthetic glycolipid modification of red blood cell membranes" was not until 2007 (Frame *et al.*, 2007, Table 3) and this paper described a range of Kode FSL blood group constructs including FSL-A, -B, -Le^a and -FLRO4, although at the time of writing the terminology for FSL constructs was SYN (for synthetic), and the terminology "kodecyte" had not yet evolved (see Preface). This journal paper was preceded by four conference papers in 2005 and 2006 (Henry *et al.*, 2005; 2006a; 2006b; Frame *et al.*, 2006), and the patent application "Synthetic membrane anchors" Figure 11 (WO 2005/090368). Furthermore, this publication was preceded by the RCPA Educational Survey Exercise trials which were undertaken much earlier in March 2004 (although not published until 2009, Henry, 2009), and the CSL Biosciences ABO sensitivity control commercial product called Securacell™ (Figure 13), which was market released in February 2005 (Chapter 3. Synthetic Glycolipids – Second Generation Kode Technology).

This foundational paper (Frame *et al.*, 2007) describes the ability of FSL-A and -B trisaccharide constructs to quantitatively modify red blood cells with A & B antigens. Additionally, it highlighted the ability of FSL constructs to probe deeper than natural glycolipids, because it could create naturally impossible red cells. For example, the construct FSL-acquired B was used to modify group O cells, and thus create cells that had acquired B antigen in the absence of A antigen (in nature the acquired B antigen is made only on group A red cells where some of the A antigen is damaged (deacetylated) into a B-like antigen). These acquired B kodecytes, bearing only an acquired B antigen, were thus able to dissect antibody responses previously not possible, and determine the acquired B activity of anti-A,B reagents. Similarly, Lewis kodecytes were created where the red cell had only Le^b antigen, again something not possible in nature (see Chapter 1. Lewis Blood Group Glycolipids: Origins of Kode Technology).

The first Kode technology based product, Securacell™ (Figure 13) which used FSL-A and FSL-B trisaccharide antigens attached to group O red cells, was part of a new quality control product developed by CSL Biosciences after several years of product development in collaboration with the author and his team at Kode Biotech. This product was released to the market following the successful outcomes of the March 2004 Royal College of Pathologists Australasia (RCPA) Educational Survey Exercise where 310 Australasian laboratories undertook blind evaluation of kodecytes. After 18 years of being continuously in the market Securacell™ is still the only qualitative ABO control product in existence.

Figure 13. First Kode Technology based product, an ABO sensitivity control product Securacell™. Introduced in the Australian market on February 10th 2005 CSL. Soon after a second variation (containing 2 instead of 4 tubes) of the product called Securacell™ Light was also released. The Kode technology aspect of this quality control product has controlled low levels of A and B antigens on the kodecytes, creating a cell with an A_wB_w cell phenotype. This product was sold by CSL (Seqiris IH), but the product line was acquired and is now sold by Immulab, Paragon Care.

This RCPA educational exercise survey was repeated in 2008 and a summary of the research from both surveys were published in the paper "Modification of red blood cells for laboratory quality control use" (Henry, 2009). This paper showed the value of kodecytes to both identify potential deficiencies in procedures and personnel, but also the ability of them to be able to improve outcomes when implemented. A direct consequence of the results was that several monoclonal reagents on the market were reformulated to a higher standard, after customers had complained that those reagents were not performing well against the Securacell™ product. A relevant quote from that paper is

> "*Surprising was the fact that 6% (2004) and 3% (2008) of assays recorded a negative result with a further 17% (2004) and 12% (2008) recording weak scores (w or 1) for the strong A-kodecytes. In both years this was predominantly attributed to the poor performance of a batch of product in a single technology platform. Of similar concern was 18% (2004) and 9% (2008) of assays recording a negative result for the medium A-kodecytes. Unsurprising was that the weak A-kodecyte sample was only detected by 28% (2004) and 36% (2008) of laboratories.*
> *Despite there being apparent improvement over the 4-year period these trials clearly identified a significant inability of some centres, staff or technologies to detect red cells expressing significant levels of ABO antigen."* (Henry, 2009)

However, the Securacell™ product was not without its deficiencies and over time it was found that some confirmed quality reagents, were not performing as expected against kodecytes. This started an investigation

into the performance of the kodecytes, and it was later found that some quality monoclonal antibodies do not like trisaccharide blood group A or B antigens, yet react as expected with tetrasaccharide antigens (Barr *et al.*, 2014). This novel and unexpected observation resulted in a change of the FSL blood group antigens on the Securacell™ products from trisaccharide to tetrasaccharide blood group antigens. Additionally in 2011 the first glycan-CMG spacer constructs were synthesised (they were specifically made to evaluate this specificity issue) and they were found to have better performance than adipate spacer versions. As a consequence in 2013 the Securacell™ product was upgraded to the 3rd generation CMG constructs (with tetrasaccharides), and thereafter the product has performed to expectation with all reagents and without any issues.

The trisaccharide to tetrasaccharide and adipate to CMG spacer observations were later documented in the "Mapping the fine specificity of ABO monoclonal reagents with A and B type-specific FSL constructs in kodecytes and inkjet printed on paper" publication (Barr *et al.*, 2014). This important paper, as well as resolving the trisaccharide to tetrasaccharide issue, also introduced the concept that the same FSL constructs used to make kodecytes could also be attached to surfaces and used in enzyme immunoassays (EIA). This publication also demonstrated that FSL constructs could be dispersed in PBS and inkjet printed as words onto paper, then be reacted with a primary antibody and when the EIA was done, the printed characters would appear where the primary antibody had attached to the FSL antigen. This EIA methodology and examples are explained in detail in both the journal publication (Barr *et al.*, 2014) and in Kode Technology Illustrated Technology Manual (Appendix 2, Henry *et al., 2023*) and also see below (Mapping antibodies specificities with kodecytes and koded surfaces) for further examples. This paper also found that ABO antigens present on koded solid surfaces in an EIA method do not always show the same specificity range when the same as antigen is present on cells (e.g. kodecytes). This observation although largely unappreciated is very important for researchers using monoclonal reagents (formulated for red cell phenotyping) against samples they were not formulated for (e.g. tissue immunohistochemistry), and has also been demonstrated earlier with Lewis reagents against natural glycolipids (Henry *et al.*, 1995a) and with FSL-Lewis constructs (Frame *et al.*, 2007, Williams *et al.*, 2016a).

Another paper "Flow cytometry evaluation of red blood cells mimicking naturally-occurring ABO subgroups following modification with variable amounts of FSL-A and B constructs" (Hult *et al.*, 2012) demonstrated the ability of kodecytes to mimic weak subgroups of A. This paper and its preceding abstract (Hult *et al*., 2008), using trisaccharide A and B FSL constructs, compared kodecyte flow cytometry with serology and compared the reactivity of kodecytes made over a range of antigens concentrations with natural weak subgroups (e.g. A_x). The key observation from this paper was

> *"Using appropriate concentrations of FSL-A and -B constructs, kodecytes could be easily created that gave manual tube serology and flow cytometry profiles identical to those observed for ABO subgroups expressing low levels of A and B antigens."* (Hult *et al.*, 2012)

The importance of this paper was that it shows that a user can precisely modify the amount of antigen added to a red cell by simply changing the concentration of FSL in the transformation solution and thus make mimics of natural cell serologic reactions. Because cells of the weak blood group antigen phenotypes are very rare (typically much less than 1:1000) and sourcing them for use as controls is logistically difficult, the ability to

simply make a serologic mimic of these weak subgroups at will, by simply adding a solution of FSL constructs to group O cells is an appealing concept for both training serology and quality control.

This ability to use kodecytes to mimic serological reactions was extended beyond quality control and was also used for making serologic panels for training students (Perry & Henry 2012a; 2012b). The first paper on this topic was published in 2015 "Training students in serologic reaction grading increased perceptions of self-efficacy and ability to recognise serologic reactions but decreased grading accuracy" (Perry & Henry, 2015), however the practice of using kodecytes for training students had been in continuous use at the author's university for several years prior. The observations from the paper on using kodecytes to teach students serologic reaction grading showed that although the technique increased self-confidence and ability of the students to recognise serologic reactions, it surprisingly decreased their accuracy in grading. A pertinent summary of the outcomes of this paper are

> "Thus, there appears to be value in using cells with weak serologic reactions, but the value may not necessarily be in the ability to change behavior, but rather in the ability to recognize and monitor those with serologic grading difficulties." (Perry & Henry, 2015),

This first paper used IgM reactions caused by ABO antibodies reacting with A weak, B weak kodecyte, as did not address indirect serologic reactions caused by IgG and detected with anti-immunoglobulin reagents. The second generation of this technique used FSL peptide antigens that reacted with IgG and is discussed below (see Kode Technology Theme: Peptides & Proteins).

Mapping antibodies specificities with kodecytes and koded surfaces

The ability to inkjet print FSL antigens onto paper (Barr *et al.*, 2010; 2014; WO 2011/002310) and then use them to define the specificity of polyclonal and monoclonal antibodies was a very useful method development. As can be seen in Figure 14 the method allows the user to print the name of the antigen as characters onto paper, and to print a range of different antigens into the same well/slot. When tested by EIA against different reagents the antigens that antibody reacts with appear as characters, while the antigens it doesn't detect remain invisible. Furthermore the area outside the printed area acted as a non-specific reagent binding control, and typical blocking steps are not required. Using this EIA technique and the ability to also compare results with cell bound antigen (e.g. kodecytes) a variety of different antibodies were glyco-mapped for specificity, and two papers soon followed.

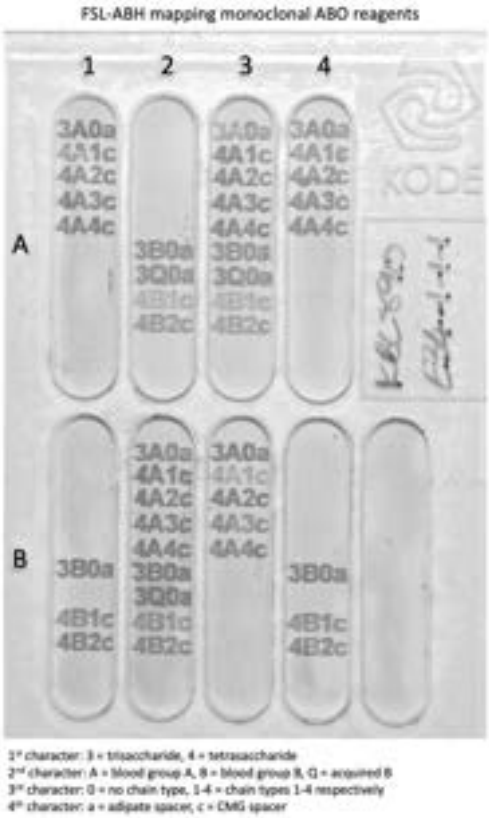


Figure 14. Mapping antibodies specificities with inkjet printed FSL constructs. In this image various FSL ABO constructs are printed on paper and reacted with serological ABO reagents by EIA (Barr *et al.*, 2014). Every reaction well-slot on the same plate has the same FSL constructs printed in it, and is tested against a different reagent. The identity of the FSL only becomes visible when it reacts with antibody (and is subsequently visualized with an anti-Ig enzyme conjugate and a precipitating chromogenic substrate. In all examples the printed characters identifies the FSL specificity, including in the ABO example the type of FSL spacer used. Unseen in all reaction wells are the printed FSL negative controls, and PBS print control. Interpreting the results simply requires deciphering the visible text. For example, the ABO monoclonal reagent in well A1 is an anti-A which reacts with all chain types of blood group A, while the reagent in B1 is an anti-B which react with B type 1 and B type 2 but does not react with acquired-B (in contrast to the acquired-B reactive (3Q0a) anti-B reagent in reaction well A2).
Image reproduced from Henry *et al.*, 2023.

The "Glycomapping the fine specificity of monoclonal and polyclonal Lewis antibodies with type-specific kodecytes and FSL constructs on paper" (Williams *et al.*, 2016a) involved and extensive mapping of the specificity of monoclonal and polyclonal Lewis reagents, and required the synthesis of a range of new and complex FSLs including the pentasaccharides of ALe^b, BLe^b, ALe^Y, and BLe^Y. The article "Function-Spacer-Lipid constructs of Lewis and chimeric Lewis/ABH glycans. Synthesis and use in serological studies" details this synthesis (Ryzhov *et al.*, 2016).

In this paper both kodecytes and inkjet printed FSL constructs were used to dissect the fine specificity of monoclonal and polyclonal reagents. As iterated before the Lewis kodecytes have only a single Lewis antigen, unlike natural cells which usually have multiple Lewis antigens, and their precursors. Natural red cell phenotypes were only able to classify the monoclonal reagents as having two specificities while kodecytes were able to define 6 unique specificities. Similarly natural red cell phenotypes were only able to classify the polyclonal reagents/serum samples as having two specificities while kodecytes were able to define 5 unique specificities. This ability to define the fine specificity of monoclonal reagents is important, as not only can it determine the potent value of a clone, but it will also guide interpretation of results, especially when used in immunohistochemistry.

The other paper "Monoclonal anti-A activity against the FORS1 (Forssman) antigen" (Barr *et al.*, 2015) mapped the cross-reactivity of monoclonal anti-A reagents for the newly discovered (by the author and his colleagues Svensson *et al.*, 2013) FORS1 the 7th human carbohydrates blood group system. This research explained why some anti-A reagents reacted with a blood group system unrelated to the ABO system (and why the extremely rare blood type FORS1 was incorrectly thought to be the A$_{pae}$ subgroup). This research required synthesis of FORS1 FSL constructs (the synthesis of which was later published Popova *et al.*, 2019)

so that FORS1 serologic mimics (FORS1-kodecytes) could be made. These FORS1 constructs were made available to other investigators to make FORS1-kodecytes who then used them to measure the prevalence of antibodies to this new histo-blood group system (Jesus *et al.*, 2016; Hult *et al.,* 2018). It is also of note that EIA used both cellulose paper and PA66 nylon nanofibers (WO 2015/084187) as substrates for printing the FSL constructs on.

This ability to attach FSL-glycan constructs to almost any surface was also explored in depth (Barr *et al.*, 2016; Williams *et al.*, 2016b). In the "Ultra-fast glyco-coating of non-biological surfaces" paper it was shown that

> *"the FSL constructs when optimised could in a few seconds glycosylate almost any non-biological surface including metals, glass, plastics, rubbers and other polymers. Although the FSL glycan coating was non-covalent, and therefore temporary, it was sufficiently robust with appropriate selection of spacer and surface that it could capture anti-glycan antibodies, immobilize cells (via antibody), and withstand incubation in serum and extensive buffer washing, making it suitable for diagnostic and research applications."* (Williams *et al.*, 2016b)

Of the 70 different non-biological and natural fibre surfaces evaluated, including hydrophobic and hydrophilic surfaces, no surface could be found in which the FSL did not adhere. It was noted however, that although a coating would occur within seconds, for some surfaces a stable coating required 30 minutes contact.

The importance of these observations were that, at this stage there was no known biological or non-biological surface that could not be coated with an FSL (WO 2014/007649), although in 2018 it was observed that yeast (excluding their bud scars which did label) would not efficiently label with FSL constructs (Raghuraman, 2021).

Antibody quantitation

The first journal paper on kodecyte antibody quantitation "An improved Fc function assay utilising CMV antigen coated red blood cells generated with synthetic Function-Spacer-Lipid constructs" used kodecytes to quantitate functional IgG activity in intravenous immunoglobulin (IVIg) preparations (Georgakopoulos *et al.*, 2012). This research included an evaluation of the glycan-bearing FSL constructs Galili (Galα1-3Galβ1-4GlcNAcβ), Galα1-4GlcNAcβ and GalNAcα1-3Galβ and found that these FSL constructs at very high concentrations on kodecytes, in the presence of functionally active IgG, were able to activate the complement cascade and cause hemolysis. However ultimately this paper found that a FSL-CMV peptide was a better choice than FSL-glycans for this IVIg assay (see Kode Technology Theme: Peptides & Proteins).

The ability to kodecytes to react appropriately with antibody was well established, and it was noted by the author from earlier work with monoclonal antibodies that the performance of a reagent to detect low levels of antigen was related to their titre; that is the higher their titre the more likely they would be to detect a weak subgroup (Le Pendu & Henry, 2002). In many respects this concept seems counter-intuitive, because why for example would a cell with 100 antigens, react stronger with a reagent that had 10,000 antibodies than it would against a reagent that had 1,000 antibodies (i.e. a reagent with 100 × more antibody than available antigen performs better that a reagent with 10 × more antibody than available antigen). This concept was

potentially resolved by the author and is explained in the publication “Kodecyte: modifying the surface of red blood cells” (Henry, 2020) and became the underlying principle of using kodecyte to quantitate antibody (without the requirement of dilution). The principle is explained as follows (but also see Figures in the publication for clarity);

> *“It is speculated that the mechanism of action in the kodecyte assay is based on the measurement of the chance at equilibrium of a collision between antibody and antigen resulting in a complex (Ag + Ab ⇄ AgAb), and this event is directly affected by antigen density. With a natural cell, having a very high density of antigen, it is almost always able to interact with all the antibody present (i.e. a collision between an antibody and its corresponding antigen is almost certain). Thus, in the case of titration (serial dilution) antibody can be diluted to very low concentrations and it will always have a very high probability of colliding with an antigen (Fig. 2). In contrast, random collisions of antibody with the low density of antigens on kodecytes are much less, and most antibodies present in a sample will not collide with an antigen (Fig. 3). The lower the antigen density becomes the larger the amount of antibody required to allow a random collision to occur, and hence the direct relationship of kodecyte antigen levels with concentrations of antibody in undiluted plasma.”* (Henry, 2020)

Typically methods to quantitate antibody levels involve dilution of the plasma samples until they become non-reactive, then they use this dilution end-point to assign a relative level of antibody to a sample. In contrast the kodecyte antibody quantitation method “A standardized kodecyte method to quantify ABO antibodies in undiluted plasma of patients before ABO incompatible kidney transplantation” (Perry *et al.*, 2019) is technically able to create “antigen dilutions” on cells (kodecytes) (Perry & Henry, 2016; Henry, 2020). This paper used the blood group A type 2 glycan antigens to quantitate ABO antibodies. Kodecytes were able to be tuned to have levels of antigen in them which were reactive only when an antibody level exceeded a specific level. As a consequence, a simple kodecyte panel could be created that only reacts when an antibody (in an undiluted sample) exceeds a specified level, and thus assay is able to quantitate antibody. For example a three cell kodecyte panel with a strong medium and weak antigen levels would all react with a sample with high antibody levels, while only the strong and medium kodecytes would react with a medium level antibody and only the strong kodecyte would react with a low level antibody. Where these levels are set is defined by the level requirements of the assay and then adjusting the kodecyte antigen levels to react appropriately. The publication found a high level of correlation between the accepted dilution method and the kodecyte method (Perry *et al.*, 2019). Although it is not yet established if the kodecyte method is better than the dilution method, the author believes that the kodecyte methodology, as it more closely resembles the state of native plasma (i.e. not diluted) of a patient, is possibly a more accurate *in vitro* assessment of a potential *in vivo* antibody response (although still remains to be proven).

A second paper involving kodecyte measurement of antibody levels was reported in “ Incidence in plasma of low level antibodies against three xenotransplantation and immunotherapeutic glycan antigens” (Perry *et al.*, 2020). This research measured the human plasma levels of antibodies (as undetectable, low, medium or high) against the xenotransplantation and immunotherapeutic glycan antigens Galα1-3Galβ1-4GlcNAc (Galili), GalNAcα1-3Galβ1-4GlcNAc and Rhaα. Antibodies against these antigens are of clinical relevance, particularly those against Galili which has a range of known clinical sequalae and was also at the time in clinical trials as in immuno-oncotherapeutic (see below, A potential immuno-oncotherapeutic). This research found that:

> "Results demonstrate independence between antibody specificities and substantial variation between individuals in levels of these antibodies, with >92% of the population having medium or high levels of at least one specificity. However, of particular importance was that 5–8% of the population had low levels of both IgM and IgG to at least one specificity and these individuals would probably have a poor immediate response when challenged by the corresponding antigen."(Perry *et al.*, 2020)

and the potential implications of these results to xenotransplantation and immune therapeutics (Galili, 2021) are important.

Hemolysis

The paper "An improved Fc function assay utilising CMV antigen coated red blood cells generated with synthetic Function-Spacer-Lipid constructs" (Georgakopoulos *et al.*, 2012) demonstrated that FSL-glycans on kodecytes were able to activate the hemolytic complement cascade. Additionally research with modelling transfusion reactions with kodecytes had also demonstrated the ability of incompatible kodecytes to activate the complement cascade (Oliver *et al.*, 2011b) (see below, Modelling transfusion reactions and antibody neutralization).

Complement and xeno antibodies levels (Perry *et al.*, 2020) are both important to the Kode technology based immuno-oncotherapeutic in development (see below, A potential immuno-oncotherapeutic). It is also well known that the quality of *in vitro* results is highly dependent on the quality of the samples and reagents used, and an evaluation of the literature identified that although the plasma protein complement had clearly defined storage parameters, these were somewhat arbitrary and based on highly variable historical methodology, and disparity between different authors. As a consequence the authors decided to determine if well-tuned kodecytes would have the potential to more accurately define the stability of complement in storage, and thus reduce variables associated with research involving the immuno protein complement.

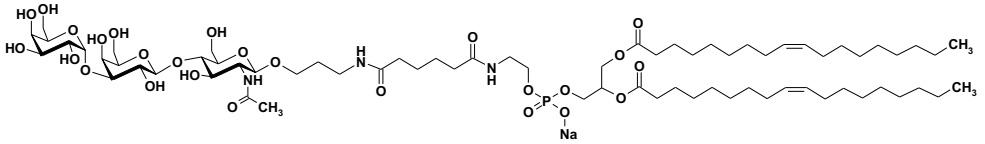
In the paper "Antibody complement-mediated hemolytic studies with kodecytes reveal human complement utilized in the classical pathway is more stable than generally accepted" (Perry *et al.*, 2016) kodecytes were prepared using the xeno (heterophile) antigen Galili (Galα1-3Galβ1-4GlcNAc) which gave standardised hemolytic reactions with a standardised serum sample (Perry & Henry, 2016). Serum samples were then subjected to range of time and storage temperature variations, and the level of complement activity in the samples was then measured. In most storage scenarios complement was found to be more than twice as stable as generally accepted. The method also provided an alternative and more simplistic way of determining complement activity in serum and without the requirement to source sheep (heterophile) blood, required by the historical assay. The extension of his work also showed that FSL-Galili when printed on paper was able to activate the complement cascade and be detected by EIA methodology (Perry, 2014) and it can be used to train students to recognise hemolytic (hemolysis) reactions (Perry & Henry, 2013).

A potential immuno-oncotherapeutic

The story on how Kode Technology became an immuno oncotherapeutic product is somewhat unusual. An aspect of the story dates back to when the author was working in Sweden at Göteborg University (Figure 6) and where xenotransplantation of pig organs into humans was being actively researched. During this period the important xeno-antigen Galα1-3Galβ1-4GlcNAc was discovered by Dr Uri Galili (and also independently by the team in Sweden). This antigen was colloquially known then as the Galili antigen. When the construct FSL-Galα1-3Galβ1-4GlcNAc constructs were first made in 2005, for want of a simple name, they were called FSL-Galili (after Dr Uri Galili). These FSL constructs from 2010 were sold by the R&D company Sigma-Aldrich (under a distribution & license agreement), as FSL-Galili(tri) F9432 (Figure 15).

FSL-Galili(tri)

FSL-GALILI(Galα3Galβ4GlcNAcβ)-SA1-L1



Molecular Weight = 1478.74
Molecular Formula = C70H125N3NaO26P

SIGMA-ALDRICH

Product Information

FSL-Galili(tri)

Catalog Number **F9432**
Storage Temperature –20 °C

Synonym: FSL-GALILI(GALα3GALβ4GLCNAcβ)-SA1-L1

Product Description
Molecular formula: $C_{70}H_{125}N_3NaO_{26}P$
Molecular weight: 1478.71

FSL-Galili(tri) is a KODE™ technology construct designed to label hydrophobic surfaces including living cells with the ubiquitous animal antigen Galili. Human cells are Galili antigen negative. All KODE FSL constructs consist of three essential designable features:

- functional component (F)
- spacer (S)
- [illegible]

Precautions and Disclaimer
This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions
A Stock Solution is prepared by reconstituting the product at a concentration of 2 mg/ml in saline or PBS. Buffered solutions are preferred for long-term storage. The product should not be reconstituted in water, unless used immediately as the product is unstable when stored in water.

The 2 mg/ml Stock Solution can be frozen in aliquots for later usage. Thawed product should be briefly sonicated before use. The Stock Solution can be diluted

Figure 15. Schematic of structure and Product Information for FSL-Galili. This antigen has the trisaccharide structure Galα1-3Galβ1-4GlcNAc. This construct is the active pharmaceutic ingredient (API) in the immuno-oncotherapeutic product known as AGI-134 (and termed as αGal-BOEL in the patent (WO 2015/170121) (Figure 18). A copy of the 2014 product information sheet relating to the Sigma-Aldrich product FSL-Galili is also shown. https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/423/002/f9432dat.pdf

In 2014 while the author and his team were working with controlling FSL-Galili mediated complement lysis of red cells, unbeknownst to the author, Dr Uri Galili (and the company Agalimmune Limited), were attempting to use natural Galili glycolipids isolated from animal tissues as an immuno-oncotherapeutic agent (Galili *et al.,* 2007; Galili, 2021). (see also the authors experiences associated with preparation of natural glycolipids, Chapter 2. Glycolipids – First Generation Kode Technology).

As it happened Uri Galili while looking in the Sigma-Aldrich catalogue noticed his name (FSL-Galili), and realised the product F9432 (Figure 16) was a synthetic glycolipid version of the natural glycolipid they had been using. Agalimmune then purchased product from Sigma-Aldrich, found it worked well, and soon after made contact with Nicolai Bovin, a long-term colleague of his. The following are related email exchanges.

Subject: Uri Galili
Date: Saturday, 5 April 2014 at 5:30:22 AM New Zealand Daylight Time
From: Бовин Николай
To: Stephen Henry

Hi, Steve.
I had a talk with Uri Galili and Paul Hatala from Agalimmune.
They have our “Galili” FSL from Sigma. Looks that they were happy with this material in anti-cancer research and now think about scaling up. And in parallel, - how to improve the molecule
Best wishes
Nicolai

Subject: KODE Biotech
Date: Wednesday, 9 April 2014 at 12:35:37 PM New Zealand Daylight Time
From: Stephen Henry
To: Paul.Hatala@agalimmune.com
CC: uri.galili@agalimmune.com, Niclolai Bovin, shenry@kodebiotech.com

Hi Paul/Uri,
Please let me introduce myself and KODE Biotech. I understand from my colleague Nicolai Bovin that you have been finding success with the FSL-Galili construct in your research. As you are probably aware Nicolai and I have been working on KODE technology now for many years and have found these function-spacer-lipid construct to be very useful.
I look forward to being able to share with you further information on some of the surprising things the FSL constructs can do (when we put an NDA in place). Perhaps one of immediate interest to you is that we are using FSL-Galili to control complement mediated lysis – it does this very well.
If you are thinking of scaling up your needs for FSL-Galili constructs or to have improved variations then we should establish a proper R&D license (with commercial options) and an NDA sooner rather than later.
In the meantime you may find the publicly available information about KODE Technology at www.kodecyte.com of some interest – in particular the slideshare presentation
With best regards
Steve.

In brief, FSL-Galili was found to perform better than the natural glycolipid and synthesis was scalable. This resulted in Agalimmune Limited licensing the technology from Kode Biotech in 2015 (see press release, Figure 16 and patents Figure 17). Although this is an important asset in the Kode Technology portfolio it is of note that the invention of this immuno-oncotherapeutic was independent of Kode Biotech and the author.

FOR IMMEDIATE RELEASE
15 June 2015

agalimmune

Agalimmune Licenses KODE Biotech's FSL Technology for Development of Cancer Immunotherapeutics

Deal worth up to $31M USD (£21 million GBP)

London, UK, 15 Jun 2015: Agalimmune Ltd, a biopharmaceutical company focused on the development of a pipeline of innovative immunotherapies for the personalised treatment of cancer, announced today it has licensed KODE Biotech's Function-Spacer-Lipid (FSL) cell surface membrane modification technology (KODE™ Technology). The proprietary technology will be incorporated by Agalimmune into its novel cancer immunotherapy molecules.

Under the terms of the agreement, KODE may receive up to $31 million USD (£21 million GBP) in development and sales milestones, plus royalties on net sales. Agalimmune has exclusive rights to the use of the technology in the field of intratumoral injection for cancer treatment, and specifically relating to the clinical candidate molecule AGI-134. KODE™ Technology has been extensively validated by Agalimmune prior to in-licensing, and has been shown to be effective for treatment of both primary and secondary tumours in animal models.

Agalimmune injects synthetic animal-antigen mimetics, which incorporate the FSL constructs, into primary tumours. As the immune system naturally rejects animal tissue, it attacks the modified tumour. In this process of destroying the tumour, the body's immune system is educated to recognise the patient's own (autologous) tumour antigens and so destroy unmodified primary and secondary tumours. http://www.agalimmune.com/index.php/moving. KODE™ constructs have been engineered to be easily dispersed in biocompatible media, spontaneously incorporate into cell membranes, and have low toxicity.

Figure 16. An image of the license deal press release from Agalimmune Limited. This 15 June 2015 document announces the licensing of KODE Biotech's Function-Spacer-Lipid (FSL) cell surface membrane modification technology (KODE™ Technology).

WIPO | PCT

WO 2015/170121 A1

WIPO | PCT

WO 2017/082753 A1

Figure 17. The two original FSL-Galili patents and its use as a cancer therapeutic. It is of note that on the first application (WO 2015/170121) the inventorships is independent of Kode Biotech and its associates. However, on the second patent (WO 2017/082753) the author and Nicolai Bovin and members of his synthesis team are listed as inventors.

Once the technology was licensed (more correctly an R&D agreement with embedded commercial licensing option was executed) a significant collaborative R&D program was initiated, which involved designing and evaluating a range of third-generation FSL-Galili constructs including CMG linear and branched variations. These improvements were patented (Figure 17), however as animal trials were producing good results it was the second-generation adipate FSL version (Figure 15) that progressed to human clinical trials. In 2017 Agalimmune Ltd was acquired by BiolineRx, an Israeli late clinical-stage biopharmaceutical company focused on oncology, and they completed phase 1/2a multicenter, open-label study human clinical trials in 2022. For more information on this Phase 1/2a study see https://clinicaltrials.gov/ct2/show/NCT03593226.

On December 20th, 2022 BioLineRx announced the results from the Phase 1/2a clinical trials. (The full report is also available at https://ir.biolinerx.com/node/13171/pdf). In summary

- the Phase 1/2a study of intratumoral cancer vaccine candidate, AGI-134, was designed to evaluate the safety and biological activity of AGI-134 in patients with unresectable metastatic solid tumors.
- The study met its primary endpoint of AGI-134's safety and tolerability.
- Part 1 demonstrated that AGI-134 was safe and well tolerated, with no dose-limiting toxicities reported.
- There was observed initiation of immune activity in patients. T cell and macrophage tumor infiltration was seen in approximately one-third of evaluable patients' injected tumors, and in approximately half of evaluable patients' un-injected lesions. Radiological assessments found that 29 percent of patients in the trial achieved best overall response of stable disease. Seven of the 11 patients who achieved stable disease had previously failed checkpoint-inhibitor therapy.

The author also wrote to Dr Uri Galili who was not a part of these clinical trials, for his opinion on the results reported by BioLineRx. His verbatim response is as follows.

"Hi Stephen
Thank you for the publication. I think the reported results look very encouraging. Evidently, the fact that the drug is safe for use and well tolerated even at the dose of 200mg implies that toxicity may not be an obstacle in the future. I find the main encouraging issue, the observation of APC and cytotoxic T cells in un-injected metastases in half of the treated patients. This suggests that the AGI-134 succeeds in "forcing" the immune system to recognize the tumor antigens in cancer patients as antigens to react against. Of course the extent of this immune response varies from one patient to the other and depends on the nature and number of tumor antigens, the phase of the disease and how potent is the immune system in different patients. Their results suggest that in almost half of the patients there was some kind of an induced protective immune response against metastatic cells. It is possible that such an immune response, keeps detectable metastases "at bay" and that it is effective enough to destroy small clusters of metastatic cells, therefore the observation of "stable disease" in a third of the patients. I have had no contact with BiolineRx, but it is probable that the patients they included in the study were at advanced stages of the disease and they did not respond well to standard therapies, prior to this study (e.g., patients failing checkpoint inhibitor therapy). I always claimed that this treatment may be regarded as an adjuvant therapy in addition to (rather than instead of) standard therapies. I believe that if this treatment is used in earlier stages of the disease it is likely to be even more effective. I also suggested in the past that in patients who are planned to undergo resection of the tumor, intratumoral injection of alpha-gal glycolipids 2-3 weeks prior to surgery may induce a systemic immune response against micrometastases, long after the primary tumor was resected. It is further possible that this treatment will synergize well with checkpoint inhibitor therapy (as suggested in the mouse study with AGI-134). Thus, in my opinion, these clinical trials should further progress.
Best Regards and I wish you Happy Holidays, Uri"

As at the time of writing this thesis no further public information was available on AGI-134. However, Kode Biotech the company was actively pursuing the spin-off veterinary opportunities for this technology.

Modelling transfusion reactions and antibody neutralization

Pre-1980 it was relativity common practice to give a patient with potent Lewis antibodies (almost always anti-Le^a) an infusion of random plasma from a Lewis positive individual, which would allow for blood transfusion of Lewis incompatible blood (Mollison et al., 1963; Pelosi et al., 1974; Hossaini 1972). This somewhat unique approach in transfusion practice caused the temporary neutralisation of Lewis blood-group antibodies allowing for a safe but technically incompatible blood transfusion. There were never signs of a hemolytic transfusion reaction, either immediate or delayed. However, the global epidemic of HIV/AIDS (human immunodeficiency virus infection and acquired immunodeficiency syndrome) began in 1981 and the practice was not to use any blood or blood product other that absolutely required. As a consequence the practice of using plasma to neutralise antibodies was discontinued and replaced with the minor inconvenience of finding compatible Le(a-b-) blood. However, the concepts of being able to *in vivo* neutralise antibodies with an infusion of glycolipids was revisited by the author and PhD student Caroline Oliver (Oliver, 2013) using Kode Technology (Henry, 2012). Here instead of using glycolipids, FSL-blood group A constructs (as they were considered to be similar glycolipids) were used. In the first of three papers on this topic, "Modeling transfusion reactions and predicting in vivo cell survival with kodecytes" (Oliver *et al.*, 2011a) methodology and models for measuring transfused cell survival were developed. There were three primary issues, the first was that laboratory animals do not have an ABO blood group system, second they do not have ABO antibodies and third was that current methodology for accessing *in vivo* cell survival were complex and crude.

The first two issues were relatively easy to solve and that was to make blood group A kodecytes with murine red cells (the blood transfusion), and immunise the mouse with blood group A salivary antigens (the incompatible recipient). The third issue was resolved by making the blood group A kodecytes also biotin-kodecytes (i.e. A+biotin kodecytes). By looking at blood films taken post transfusion stained with an avidin fluorophore and counting the fluorescent kodecytes remaining, survival could be determined. It was found that a transfusion of Gb3-kodecytes (the control glycan as the animal does not have anti-Gb3) into an animal with or without anti-A survived normally. Similarly A-kodecytes transfused into an animal without anti-A survived normally. In contrast A-kodecytes transfused into an animal with anti-A were rapidly destroyed.

Additionally this paper described a novel proof-of-concept methodology. Here a whole blood sample (containing up to 5% A+biotin kodecytes) was passed through an avidin agarose column, which captured the kodecytes. After removing the captured kodecyte attached to the gel they were released by vortexing (presumably by pulling the FSL-biotin lipid tail out of the cell membrane, with damaging the cell). The resultant kodecytes when assessed were determined to be blood group A (as a consequence of FSL-A modification). Although this method was not used (due to the need for relative large quantities of blood, which is limited with mice, and because it only will recover compatible cells, as incompatible cells are destroyed) the method was very suitable to recover pure cell populations many hours post infusion from the circulation of an animal. Such method also has the potential to be extended to the study of *in vivo* cell biodistribution.

In the second paper, “In vivo neutralization of anti-A and successful transfusion of A antigen incompatible red cells in an animal model” (Oliver *et al.*, 2011b) the methods developed in the first paper were applied and the survival of incompatible blood transfusions, with and without prior antibody neutralisation with FSL-A constructs were determined. It was found that an infusion of FSL-A was able to completely neutralise *in vivo* antibody, and that most of the transfused cells survived normally (reminiscent of the Lewis plasma neutralisation of antibody first reported by Mollison et al., 1963). In contrast if the anti-A was not neutralised most of the transfused cells were rapidly destroyed (although the animal had no visible symptoms).
There were several intriguing and novel observations in this paper. The first was, if the FSL-A dose infused was high enough, all the circulating cells in the recipient would also become blood group A antigen positive (reminiscent of the transfusion induced transformation of A blood type first reported by Renton & Hancock, 1962). Secondly this so-called *in vivo* transformation effect lasted for about 24-48 hours and was the same whether the animal had anti-A present or not. There was no evidence of cell destruction in any animal, regardless of the presence of anti-A, and anti-A levels returned to normal soon after the acquired A antigens disappeared from the cells. The third observation was that if these previously FSL-A infused antibody neutralised animals were rechallenged with incompatible kodecytes (but this time without a pre-infusion of FSL-A) then there were two outcomes. In one group the incompatible kodecytes survived normally and in the other they were rapidly destroyed. Further (post-mortem) analysis revealed that the group with surviving A-kodecytes had much lower antibody levels that those whose A-kodecytes were rapidly destroyed. Although this work was not taken further the author speculated that

> "*Finally it is also possible that FSL constructs may in some circumstances be able to down regulate the immune system by inducing auto regulation, thereby causing down regulation of specific antibody production. The Cluster IV(ii) mice potentially showed this effect, which is also supported by the literature where prolonged immunization with glycolipids has resulted in decreased specific antibody titers.”* (Oliver *et al.*, 2011b)

Unfortunately this very intriguing finding was unable to be followed up by the author.

The final paper of the series “Modeling transfusion reactions with kodecytes and enabling ABO-incompatible transfusion with Function-Spacer-Lipid constructs” (Henry *et al.*, 2012) was a review article and provide a useful *summary of this above methods and results:*

> *“FSL constructs were used to both attach incompatible antigens onto mice red cells and also the label (FSL-biotin) to determine survival (and potentially to recover transfused cells) [30]. By stimulating hyper-immune anti-A in mice and then transfusing them with incompatible A+biotin kodecytes made from murine red cells we were able to model hyperacute anti-A mediated in vivo red cell destruction. By applying the historical concept that glycolipids can neutralise circulating antibodies, using FSL constructs, we were able to neutralise circulating anti-A and enable the in vivo survival of an incompatible transfusion of A kodecytes. Extrapolation of these results suggests that it may be possible to use FSL constructs to neutralise circulatory antibodies and allow for incompatible transfusion／transplantation although further research is still required”* (Henry *et al.*, 2012)

A patent “Method of modifying the immune response “ was also filed on this opportunity, (WO 2010/039049). The abstract reads:

> "Methods of neutralising circulating antibody and mitigating the risk of clinically significant adverse responses to incompatible transfusions and transplantations are described. The methods comprise the administration to the subject of dispersible antigen-lipid constructs."

Microbiological applications

In 2011, in collaborative research done at the Mayo Clinic with the group of Dr Stephen Russell, it was first observed that virions could be labelled with FSL constructs and still maintain their ability to infect cells (Hadac *et al.*, 2011). However this work did not involve the use of FSL-glycans to label the virus (but instead used FSL-tyrosine-^{125}I & FSL-fluorophore). Although FSL-glycan labelling could be easily done, to-date there are no publications where virions have been labelled with FSL-glycan constructs (in contrast to a large number of publications modifying virions with FSL-fluorophores and biotin (Kode Technology Theme: Fluorophores & Biotin; Appendix 2; Henry *et al.*, 2023) and FSL-PEG (Tesfay *et al.*, 2013).

Instead, cells have been labelled with FSL-glycans receptors for virions. In two publications on this topic "A synthetic globotriaosylceramide analogue inhibits HIV-1 infection in vitro by two mechanisms" (Harrison *et al.*, 2010a) and "A novel VSV/HIV pseudotype approach for the study of HIV microbicides without requirement for level 3 biocontainment" (Harrison *et al.*, 2011) FSL-GB3 (Gb_3, P^k, globotriaosyl-) was used as a receptor (Branch *et al.*, 2008; Harrison *et al.*, 2010b; 2010c). It was known at the time that the glycolipid Gb_3 was a resistance factor in HIV infection, and that HIV binds to several glycolipids *in vitro* including Gb_3. In this research FSL-GB3 was shown to be able *in vitro* inhibit HIV infection of cells and the outcomes are summarised as follows

> "In summary, we report herein a novel, soluble, completely synthetic analogue of a natural HIV-1 resistance factor, globotriaosylceramide, which we call FSL-Gb3, that has no in vivo toxicity in mice and which we have shown *in vitro* to have dual inhibitory properties. Further studies of this molecule may prove it to be a novel therapeutic for the treatment of HIV-1 infection by acting to both inhibit viremia by interacting as a soluble inhibitor with viruses present in the blood stream as well as able to insert itself into plasma membranes of Gb3-negative HIV-1 targets, converting these cells to Gb3-expressing cells capable of resisting HIV-1 infection." (Harrison *et al.*, 2010a)

> "We show that soluble Gb3 analogs inhibit *in vitro* infection of cervical and vaginal-derived cell lines by both intact HIV and the VSV/HIV recombinant virus." (Harrison *et al.*, 2011)

A patent "Carbohydrate-lipid constructs and their use in preventing or treating viral infection" was also filed on this opportunity (WO 2008/133534). The abstract reads:

> *"The invention relates to selected carbohydrate-lipid constructs and their use as mimics of ligands for receptors expressed by a virus. In particular, the invention relates to the use of selected carbohydrate-lipid constructs in methods of inhibiting virus infection and/or promoting clearance of virus from infected subjects. Carbohydrate-lipid constructs selected for use in these methods where the virus is Human Immunodeficiency Virus (HIV) are provided."*

A secondary (non-viral) observation was also reported in this paper (Harrison *et al.*, 2010a), as part of the methodology, and that was the ability of FSL-Gb_3 to bind to the Verotoxin 1 (Shigatoxin). Here is was shown by thin layer chromatography that verotoxin binds to FSL constructs as well as to natural glycolipids, and this method was used to identify the Gb_3 antigen. With the ability to use FSL constructs *in vivo* this result raise the untested but intriguing possibility to use FSL constructs to treat hemorrhagic colitis caused by Shiga toxin (verotoxin)-producing *Escherichia coli*.

In the review paper “Synthetic glycolipid-like constructs as tools for glycobiology research, diagnostics and as potential therapeutics” (Korchagina & Henry, 2015) the use for FSL-sialic acids receptors for viral attachment was first described. Here red cells were first de-sialylated with sialidase and then re-sialylated with FSL-sialyl lactosamine (SLN) constructs resulting in 6’SLN kodecytes and 3’SLN kodecytes, i.e. cells with a single sialic acid specificity. The capability of these sialyl kodecytes to bind three different strains of influenza viruses (avian H5N2/Mallard/10218, swine H9N2/9/98, and human B/HK/54800) was determined by hemagglutination. The sialyl kodecytes were able to differentiate human (3’SLN -, 6’SLN+), swine (3’SLN +, 6’SLN+) and avian (3’SLN +, 6’SLN-) influenza viruses. All viruses agglutinated native red cells and none agglutinated desialyated red cells, with these cells being the same cells as used to make the kodecytes (Korchagina & Henry, 2015).

Hyaluronic acid oligomers

The naturally occurring glycan polymer hyaluronic acid (HA) is claimed to have a number of biological functions, and one is that can enhance of embryo adhesion. In fact a commercially available embryo transfer media product exists making claim that “EmbryoGlue is a hyaluronan-enriched embryo transfer medium which aids in implantation of embryos”.

It had been previously established that embryos could be manipulated with Kode constructs and glycolipids (Blake, 2003; Carter, 2005).Therefore using a large range of FSL-HA constructs prepared from fractionated HA, murine embryos (as HA-kodecytes) were subjected to an extensive *in vitro* evaluation by PhD student Eleanor Williams (Williams, 2009; WO 2007/035116). Her thesis “Evaluation of hyaluronic acid in embryo-endometrial apposition using hyaluronic acid-lipid constructs” found that

> *“It was established that the FSL-HA constructs could quantitatively attach HA to the embryo cell membrane. In contrast, high molecular weight HA present in commercially available embryo transfer media was undetectable in treated embryos. When HA was present via FSL-HA at the embryo cell membrane, increased adhesion to endometrial cells was demonstrated in rosetting and microplate apposition assays. However, the effect was diminished when the embryos were treated as they would be clinically, i.e., with their zona intact. Toxicity assays of FSL-HA modified embryos were able to show that at appropriate concentrations was not toxic. Using FSL-HA it was demonstrated that when HA was present at the embryo cell membrane, it was capable of enhancing attachment at apposition.”* (Williams, 2009*).*

When this PhD work was extended into optimal and suboptimal embryo implantation animal models (Erikson, 2012) they were unable to conclude any biological consequences related to embryo implantation that could be attributed to FSL-HA.

The synthesis of FSL-HA was reported in detail in (Korchagina *et al*., 2012).

Kode Technology Theme: Peptides & Proteins

A total of 12 journal papers (Table 11) and 7 published conference abstracts and 3 patent applications on Kode technology associated with the author related to peptides and proteins have been published. This section is divided into three sub-themes being:

- Miltenberger & blood group systems
- Serologic teaching & training
- Microbiological applications

Table 11 Journal publications titles of author contributions related to Kode Technology Theme: Peptides & Proteins related papers. Journal published conference/meeting abstracts and patents are not listed but are included in the references. Access to copies of these articles is via Appendix 1

Reference	Article Title
Hadac *et al.*, 2011	Fluorescein and radiolabeled Function-Spacer-Lipid constructs allow for simple in vitro and in vivo bioimaging of enveloped virions
Heathcote *et al.*, 2010	Novel antibody screening cells, MUT+Mur kodecytes, created by attaching peptides onto erythrocytes
Henry *et al.*, 2011	Designing peptide-based FSL constructs to create Miltenberger kodecytes
Georgakopoulos *et al.*, 2012	An improved Fc function assay utilising CMV antigen coated red blood cells generated with synthetic Function-Spacer-Lipid constructs
Henry *et al.*, 2018	Applications for kodecytes in immunohematology
Nagappan *et al.*, 2021	COVID-19 antibody screening with SARS-CoV-2 red cell kodecytes using routine serologic diagnostic platforms
Henry, 2020	Kodecytes: Modifying the surface of red blood cells
Ryzhov *et al.*, 2021	SARS-CoV-2 peptide bioconjugates designed for antibody diagnostics
Srivastava *et al.*, 2021	COVID-19 antibody detection and assay performance using red cell agglutination
Weinstock *et al.*, 2022	Erytra blood group analyser and Kode Technology testing of SARS-CoV-2 antibodies among convalescent patients and vaccinated individuals
Henry *et al.*, 2022	Continuous population surveillance of COVID-19 immunity can be provided by blood services at low cost using routine laboratory infrastructure
Perry & Henry 2022	Simulated red cell antibody identification training panels created using SARS-CoV-2 kodecytes and immune plasma

Miltenberger & blood group systems

The Miltenberger blood group antigens are part of the MNS blood group system and are the result of genetic recombination, producing hybrid glycophorins. These variant hybrid glycophorins (vMNS) are of high incidence in East Asian populations and antibodies developing after transfusion or pregnancy can cause of hemolytic disease of the fetus and newborn and severe hemolytic transfusion reactions. As a consequence in populations where Asians are represented, antibodies against Miltenberger antigen should be screened for by antibody screening reagent red cells (Heathcote *et al*., 2011); and herein sits the technical problem. The distribution of antigenic phenotype in Asians and Europeans are often very different, for example the blood group D-negative (Rhesus) phenotype is very common in Europeans yet rare in Asians, while conversely vMNS is rare in Europeans and common in Asians. Antibodies in both systems (along with a raft of antibodies to other blood antigens) need to be screened for with reagent red cells in transfusion and pregnancy patients, but obtaining reagent cells representing both Asian and European clinically significant antigen profiles is very difficult. With the success of the synthetic glycolipid ABO sensitivity control product in

2005, interest from CSL focus turned to making vMNS (Miltenberger) kodecytes and a development program was initiated in 2004 (see Chapter 4. CMG spacers - Third Generation Kode Technology)

After 4 years of research, development and product testing (in conjunction with CSL) (Heathcote *et al*., 2008; Flower *et al*., 2008; 2011a; 2011b; Henry *et al*., 2011b) in 2009 the second license product AbtechCell™ III and Phenocell™ C, containing vMNS kodecytes was released to the market (Figure 18). Two journal papers "Novel antibody screening cells, MUT+Mur kodecytes, created by attaching peptides onto erythrocytes" (Heathcote *et al*., 2010) and "Designing peptide-based FSL constructs to create Miltenberger kodecytes" (Henry *et al*., 2011) describing this development were published.

Figure 18. AbTectcell™ II & Phenocell™ C. These are diagnostic red cells used for detection of clinically significant vMNS antibodies in patient serum. This product consists of adding Asian vMNS blood group antigens onto European red cells (kodecytes) so they can be used to detect Asian specific antibodies. This was original product was sold by CSL (Seqiris IH), but the product line was acquired and is now sold by Immulab, Paragon Care.

Other blood group systems

With the success in building an adequate vMNS blood group antigen the next natural step was to build other blood groups systems. The problem here is the blood group antigen needs to be able to be represented by a linear (non-glycosylated) epitope, and the location of the antigenic determinant (peptide sequence) on the polypeptide chain be known (Henry *et al*., 2011). The latter was a particular issue, as the general misconception is that location of the altered peptide sequence (as a result of a genetic point mutation) is the location of the antigen, which is often not correct. Instead the point mutation can induce a conformation change in the entire polypeptide, resulting the antigen determinant being remote from the point mutation. Despite this caveat, and as the actual antigenic peptides of most blood group antigens are not known (although their genetic basis is), linear epitopes covering the logical sequences flanking the peptide variation regions were made for several blood group systems including; Kell, Duffy, Kidd. None were successful, clearly indicating these peptides regions are not the antigenic determinant (unpublished).

Serologic teaching & training

The first generation of teaching kit involved the use of blood group A and B glycan antigens (see above), and reactivity was typically due to IgM direct reactions. Because the reactions were IgM they were essentially limited to teaching the grading of serologic reactions (see above Glycans), and were not suitable for creating antibody screening panels. Most serologically important antibodies (excluding ABO) are due to IgG and are detected via the indirect antiglobulin test (anti-human globulin; AHG).

During the development of microbiological peptide diagnostic assays it was observed that a significant number of candidate FSL constructs would react without apparent specificity for the target antigen, and were probably directed against (or cross-reactive) with some unknown microbiological antigen (despite the selection algorithms using the protein BLAST (Basic local Alignment Search Tool) to reduce this likelihood). These FSL-peptide reactions were caused by antibody, typically IgG with some IgM reactivity, but FSL-peptides could be found that would be predominately IgG reactive.

Although it was not known what the specificity of the antigen antibody pair was, this was not important, as the premise serology is essentially the detection of an antigen on the surface of a cell with an antibody. Therefore if an antigen can be added to a cell, and an antibody which reacts with it exists, then this antigen-antibody pair can be used to mimic normal serologic reactions (Henry, 2020).With these principles in mind a serologic teaching kit was developed, which used an FSL-antigen (of unknown specificity) and which reacted with about 10-30% of random samples. A full description of this kit (called ASKG for advance serologic kodecytes – IgG) is as follows:

> *" … can be used to make kodecytes that are predominantly IgG reactive. The Kode construct peptide antigen on these ASKG kodecytes does not represent any known infectious agent and cannot be screened for with any known diagnostic assay – it is simply an antigenic structure that will react with IgG antibodies present in about 10–30% of random donors (Note – these antibodies do not react with natural RBCs, and they only react with ASKG kodecytes). The immunological reactions on the ASKG kodecytes are real and are caused by IgG antibody reacting with cell-bound antigen (albeit one artificially attached to the RBC), Fig. 4. With the ability to control antigen concentration on ASKG kodecytes and easy access to IgG antibody, mimic antibody screening and identification (ID) panels designed for teaching purposes can be made. This involves making ASKG kodecytes from group O cells (2 h incubation at 37°C with FSL-ASKG) and arranging ASKG kodecytes and unmodified cells in patterns representative of antibody ID panels antigen profiles. Changing the order of the kodecytes and unmodified cells in a mimic ID panel will change the reactivity profile to a different antibody specificity. Similarly, changing the antigen strength of the ASKG kodecytes within an ID panel can be used to make antigenic variance and mimic reactions including dosage. IgG antibodies that react with these ASKG kodecytes are found by simply screening random samples. Initial ASKG teaching kits (https:// openrepository.aut.ac.nz/handle/10292/12731) are now under laboratory evaluation."*
> (Henry, 2020)

While developing the SARS-CoV-2 diagnostic assay (see below) and due to the high vaccination rates in the general population it was decided to change the ASK-G unknown antigen-antibody pair to a known antigen-antibody pair, being FSL-SARS-CoV-2 antigen and use COVID-19 vaccinated or convalescent plasma. The resultant outcome was a very sophisticated training kit capable of mimicking almost every known serologic reaction pattern. This outcome of this development was published in the article "Simulated red cell antibody identification training panels created using SARS-CoV-2 kodecytes and immune plasma" (Perry & Henry, 2022). Here it was found "

> *"Results: Kodecytes (positive reactions) and unmodified cells (negative) when arranged and tested in appropriate patterns in SASID panels were able to mimic IgG antibody reactions, and*

were capable of measuring both accuracy and precision in reaction grading. Conclusions: Kodecytes can be used to rapidly create in-house simulated yet realistic antibody screening and identification panels suitable for large scale training in the recognition and grading of serologic reactions." (Perry & Henry, 2022).

Microbiological applications

The first research on microbiological applications of FSL-peptide constructs was an attempt in 2007 at the development of a syphilis diagnostic assay (Patel, 2008). This early research involved evaluating a short 7 amino acid syphilis peptide (VMYASSG) either adipate or PEG_{14} linked to DOPE. To ensure solubility the peptide also had a leader sequence of GSGSG and a cysteine reside for conjugation. FSL constructs with the peptide sequence CGSGSGVMYASSG was then linked to either adipate (Ad) or PEG_{14} (Figure 19).

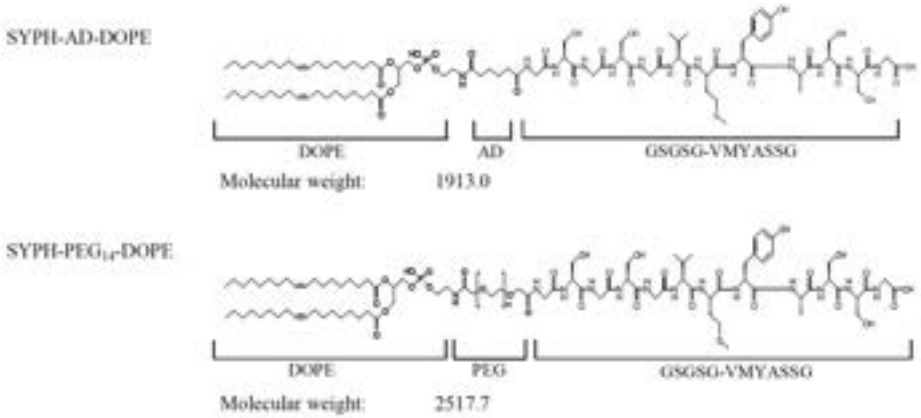


Figure 19. First attempts in 2007 at making syphilis-peptide FSL constructs. Both attempts were unsuccessful with the SYPH-Ad-DOPE construct being insoluble in saline and the SYPH-PEG_{14}-DOPE reacting non-specifically with plasma. (Image from Patel, 2008).

The SYPH-Ad-DOPE construct was insoluble in saline and unable to transform red cells. The SYPH-PEG_{14}-DOPE construct showed very poor correlation with EIA validated samples, with 26% of negative samples reacting positive and identified to be due to the PEG spacer. Essentially these first attempts to make an FSL peptide construct were failures. A few years later this peptide sequence was made into an FSL construct using the CMG spacer and this time the results were good. This research was only published as abstracts "Syphilis-kodecytes - novel function-spacer-lipid (FSL) modified red cells capable of sensitive and specific detection of syphilis antibodies" (Komarraju *et al*., 2010) and "Solid phase syphilis test utilizing KODE™ technology" (Chesla *et al*., 2010). A patent "Assays for serological detection of syphilis" was also filed on this opportunity (WO 2010/143983).

The first publication of an FSL with an amino acid functional head used in a microbiological setting was "Fluorescein and radiolabeled Function-Spacer-Lipid constructs allow for simple *in vitro* and *in vivo* bioimaging of enveloped virions" (Hadac *et al.*, 2011; WO 2012/121610). However the primary intention of that research, was to use an amino acid residue (tyrosine), only because it could be easily iodinated with ^{125}I. Because, a single amino acid does not constitute a peptide the first journal publication was instead "An improved Fc function assay utilising CMV antigen coated red blood cells generated with synthetic Function-

Spacer-Lipid constructs" (Georgakopoulos *et al.*, 2012). In this paper, FSL-glycans (see above) and FSL-peptides based on cytomegalovirus (CMV) were evaluated for their ability to cause hemolysis, as a way of determining the functional IgG activity in IVIG products. CMV was chosen as the candidate antigen because antibodies to it are of high frequency in the general population. The CMV-kodecytes were patented "Peptide-Lipid constructs and their use in a FSL-function assay" (WO 2012/118388) and are is still in current use for the validation and release of IVIG product.

A summary of the paper is as follow:

> "Fc function indices determined with CMV peptide construct–coated red cells were comparable to those obtained with the EP-based method with respect to specificity and precision. Carbohydrate-bearing FSLs revealed Fc indices lower than expected. Conclusion The use of CMV kodecytes was shown to be a convenient means of generating red cells for the determination of Fc function of immunoglobulin products and offers the possibility of significantly reducing the time required to perform this assay. (Georgakopoulos *et al.*, 2012).

Over the following years a series of antibody diagnostics for *Trypanosoma cruzi* and *Babesia microti* were also explored, and resulted in new kodecyte assays with good sensitivity and specificity (unpublished observations). In 2019 PhD student Radhika Nagappan (Nagappan, 2021) revisited the syphilis diagnostic and developed both improved algorithms for predicting FSL functional head groups, and an improved syphilis (*T. pallidum*) antibody diagnostic. The final syphilis kodecyte assay developed, when tested against syphilis EIA, screened positive and negative samples and showed that the kodecytes had a sensitivity of 98.6% and a specificity of 98% (better than almost any assay in the market). Using the refined prediction algorithms, a new antibody diagnostic assay was then successfully developed for the spirochete Leptospira. Overall, the Leptospira peptide FSL constructs detected all the serovars tested and had good sensitivity and specificity, exceeding that of existing Leptospira serological diagnostics (Nagappan, 2021).

In 2020 with the unexpected arrival of the COVID-19 pandemic, this research effort was rapidly pivoted into developing a SARS-CoV-2 antibody diagnostic (in collaboration with international colleagues). These outcomes are described in a range of publications including "COVID-19 antibody screening with SARS-CoV-2 red cell kodecytes using routine serologic diagnostic platforms" (Nagappan *et al.*, 2021); "SARS-CoV-2 peptide bioconjugates designed for antibody diagnostics" (Ryzhov *et al.*, 2021) "COVID-19 antibody detection and assay performance using red cell agglutination" (Srivastava *et al.*, 2021); "Erytra blood group analyser and Kode Technology testing of SARS-CoV-2 antibodies among convalescent patients and vaccinated individuals" (Weinstock *et al.*, 2022).

The outcomes of this research can be summarized as follows:

> "Specificity (negative reaction rate against expected negative samples) in three different CAT platforms against novel C19-kodecytes was >91%, which correlated with published literature. Sensitivity (positive reaction rate against expected positive convalescent, PCR-confirmed samples) ranged from 82% to 97% compared to 77% with the Abbott Architect SARS-CoV-2 IgG assay. Manual tube serology was less sensitive than CAT. Enzyme immunoassay results with some Kode Technology constructs also had high sensitivity. Conclusions: C19-kodecytes are viable for use as serologic reagent red cells for the detection of SARS-CoV-2 antibody with routine blood antibody screening equipment."
> "Overall, the kodecyte assay was able to achieve specificity and sensitivity at least equivalent to an established EIA antibody diagnostic. Due to the cassette design of Kode Technology, it is highly adaptable, and changing the antigenic epitope on a Kode FSL construct can be achieved within a few weeks, allowing for rapid response to new strains arising with novel antigenic mutations. Other than determining optimal concentrations for sensitivity and

> specificity, no other modifications to the methodology for use are required. This article describes an adaptable platform technology able to be easily accommodated into almost all existing transfusion diagnostic laboratories, including those with limited infrastructure, and will allow for this sector to actively participate in the screening for SARS-CoV-2 antibodies, both for population needs and therapeutic uses." (Nagappan *et al.*, 2021).

This assay was very cost-effective with 1 mg of the FSL construct able to enable more that 100,000 diagnostic tests. This point was further highlighted in the letter to the editor "Continuous population surveillance of COVID-19 immunity can be provided by blood services at low cost using routine laboratory infrastructure" (Henry *et al.*, 2022) where the cost of kodecytes per assay is stated as ½ a cent.

Later refinement of the FSL peptide resulted in an improved SARS-Cov-2 diagnostic assay requiring only a single FSL construct (rather the combination 2 constructs in the original assay) (Henry *et al.*, 2022) and it was also reworked to make serologic teaching panels (Perry & Henry, 2022) – see above. However, despite its potential for this diagnostic to be utilized by blood services to-date there has been no uptake of this diagnostic.

Kode Technology Theme: Fluorophores & biotin

A total of 6 publications (Table 12) on Kode Technology associated with the author related to FSL Fluorophores & biotin have been published. This section is divided into two sub-themes being:

- Fluorophores
- Biotin

Table 12 Journal publications titles of author contributions related to Kode Technology Theme: Fluorophores & biotin related papers. Journal published conference/meeting abstracts and patents are not listed but are included in the references. Access to copies of these articles is via Appendix 1

Reference	Article Title
Hadac *et al.*, 2011	Fluorescein and radiolabeled Function-Spacer-Lipid constructs allow for simple in vitro and in vivo bioimaging of enveloped virions
Blake *et al.*, 2011	A simple method for modifying cell/virion surfaces with a range of biological markers without affecting their viability
Henry, 2014	Magnetic bead coatings: Today and tomorrow. Chapter 5 Rapid biofunctionalization of magnetic beads with function-spacer-lipid constructs. SepMag eBook 2014 (http://sepmag.eu/free-guide-magnetic-bead-coatings)
Ilyushina *et al.*, 2014	Labeling of influenza viruses with synthetic fluorescent and biotin-labeled lipids
Henry *et al.*, 2018	Rapid one-step biotinylation of biological or non-biological surfaces
Lan *et al.*, *2012*	Fluorescent Function-Spacer-Lipid construct labelling allows for real-time in vivo imaging of cell migration and behaviour in zebrafish (*Danio rerio*)
	Lan C-C, Blake D, Henry S, Love DR. Videos relating to Fluorescent Function-Spacer-Lipid construct labelling allows for real-time in vivo imaging of cell migration and behaviour in zebrafish (*Danio rerio*) 2012; http://hdl.handle.net/10292/3475

Fluorophores

Fluorescence is a staple methodology in research, and the FSL construct FSL-fluorescein (Figure 20) which appeared the first publication on synthetic constructs (Frame et al., 2007) was developed for research purposes. This product was patented (WO 2008/030115) and is still being sold to through Sigma-Aldrich, FSL-FLRO4(fluorescein)-SA2-L1 as research product F1058.

FSL-FLRO4 has been extensively used by the author and collaborators to label cells, embryos, viruses, liposomes bacteria and surfaces (Hadac *et al.*, 2011; Blake *et al.*, 2011; Ilyushina *et al.*, 2014; Lan *et al.*, *2012;* Campbell, 2023; Carter 2007; Poudel 2020, Raghuraman 2021) and by others. A full description of the utility of this FSL and a range of other FSL fluorophores are described in detail in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al.*, 2023)

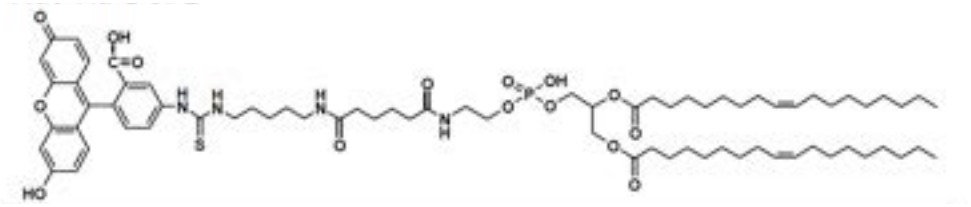


Figure 20. FSL-fluorescein (FSL-FLRO4).

Biotin

Biotin and its strong interaction with avidin is perhaps the most utilised generic non-covalent conjugation tool used in research. Early in the development of Kode constructs we identified the need for an FSL biotin construct, and a first generation version existed, in the form of biotinylated gangliosides (BioG) – see Chapter 2. Glycolipids – First Generation Kode Technology, Biotinylated gangliosides. At that time in the absence of FSL-biotin these BioG glycolipids were used in early postgraduate student research (Blake, 2003; Carter, 2007; Gilliver, 2006; Patel, 2008) to establish proof-of-concept methodology. Fortunately in 2008, following the invention of the CMG spacer, FSL biotin (Figure 21) constructs became available. Today these FSL-biotin construct are extensively sold by Sigma-Aldrich FSL-CONJ(1Biotin)-SC2-L1 as the research product F1058. Unfortunately, most researchers simply record these FSL-biotin constructs only by their product name in Materials & Methods and do not cite any publications specifically related to Kode Technology.

FSL-biotin has been extensively used by the author and collaborators to label cells, embryos, viruses, liposomes, bacteria, and surfaces (Oliver *et al.*, 2010; Blake *et al.*, 2011; Oliver *et al.*, 2011a; 2011b; Ilyushina *et al.*, 2014; Henry, 2014; Henry *et al.*, 2018b, Zalygin *et al.*, 2020; Slivka *et al.*, 2022) and by others as described in detail in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al.*, 2023).

An extensive demonstration of the features of FSL-biotin was published in Scientific Reports “Rapid one-step biotinylation of biological or non-biological surfaces” and supported by a large supplementary file (Henry *et al.*, 2018b). This paper demonstrated the ability of FSL-biotin constructs, including sterol and ceramide lipid tail variations to rapidly label almost any biological or non-biological surface, as well as anchor cells and proteins on surfaces. Other papers investigated the properties of the FSL-biotin construct and were able to demonstrate novel features including its ability to adopt extended and folded conformations (Zalygrin *et al.*, 2020).

The multiple applications and uses of FSL-biotin are described in detail in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al.*, 2023).

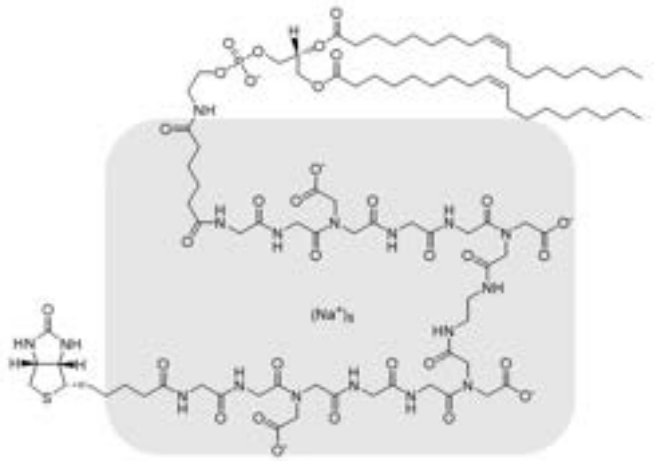


Figure 21. FSL-biotin. The FSL-biotin construct (FSL biotin-CMG(2)-DOPE) facilitates the rapid, non-covalent biotinylation of biological and non-biological surfaces, such that they are able to react with a full range avidinylated and biotinylated (via avidin) R&D probes. The grey box in the image indicates the CMG(2) spacer, with the DOPE lipid at the top

Kode Technology Theme: Other constructs

This final construct theme on "other" FSL constructs is a catch-all for a large variety of FSL constructs and concepts that do not fit easily in to the above themes, and some of which are not published in journals or patents (but are published in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al.*, 2023).

Kode Technology constructs have a cassette design of three components (function-spacer-lipid), and for each functional head group there are a range of spacers and lipids to choose from, making it possible to have multiple variation presentations of the same functional head. These FSL spacer-lipid variations and a large range of "other" functional heads on FSL constructs are listed below and described in detail in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al.*, 2023):

Variations in FSL lipids

- Sterol (WO 2012/099477)
- Ceramide (WO 2018/220603)

Variations in FSL Spacers

- FSL spacer types (WO 2009/035347)
- CMG(2) the "Goldilocks" spacer (WO 2009/048343)
- FSL TCMG triantennary spacer (WO 2016/080850; WO 2017/082753)

Variations in "other" FSL functional heads

- FSL zero (WO 2019/244138)
- FSL cyclodextrins
- FSL bioconjugation : "click-chemistry"
- FSL bioconjugation : sortase-mediated ligation
- FSL antibody-conjugates
- FSL complexons (WO 2013/081471)
- FSL charge (WO 2019/244138)
- FSL oligonucleotides
- FSL extracellular matrix (WO 2007/035116)
- FSL "bespoke" constructs (WO 2016/185331; WO 2016/072863)

Kode Technology Theme: Kode Biotech Limited

The story of the development of Kode Technology is not just about academic and scientific endeavours, it is also a story about creating a framework to enable and facilitate its development. That framework was the commercial entity Kode Biotech, whose primary purpose is to monetise Kode Technology for its shareholders and stakeholders. The interaction of business with science is often viewed with scepticism, misinformation and a perception that it results in bad (filtered) science, but the reality is without the large amounts of investment required to develop a biotechnology, it probably would not eventuate, and the fact is that science and business can work well together in a mutually beneficial cooperation. It is however, fair to say, that the development of Kode Technology was underpinned by strong commercial drivers, and focused on research likely to produce an economic product outcome. Additionally because the strategic direction was to patent, much development had to be “incubated under the radar” in order to be able to secure patent protection, before publication could be considered. Despite this a significant amount of academic investigations were undertaken (many of which were later published), via collaborations with universities and research partners.

Kiwi Ingenuity Limited – Kode Biotech Limited

The original name for the company was Kiwi Ingenuity Limited but changed its name in April 2006 to Kode Biotech Limited. Kiwi Ingenuity Limited/Kode Biotech Limited (registered March 1996; NZ Limited Company (#713905); NZBN: 9429038366413) was established by the author upon his return to New Zealand from Sweden in 1996 as a vehicle to commercialise the anticipated blood group glycolipid quality control product (Chapter 2. Glycolipids – First Generation Kode Technology). The author is the founder, CEO/Managing Director of Kode Biotech and its subsidiaries. From the outset the company has been focused on a market driven – intellectual property approach to research and all research and commercial decisions were focused on developing a strong intellectual property position that could be used to create products that would be commercially viable. Supply chain was also important for revenue generation and in 2008 the subsidiary Kode Biotech Materials Limited (NZ Limited company (#2153669); NZBN 9429032650419) was established, and in 2016 the subsidiary GlycoNZ Limited (NZ Limited company (#5893668); NZBN 9429042199526). Revenue from Kode Biotech Ltd (the IP licensor), and both supply companies (collectively termed Kode Biotech) by 2018 were generating sufficient revenue to support the companies activities.

The business model for Kode Biotech from the start was based on its acceptance of the reality that if it was going to remain a New Zealand based company (a commitment of the founder), its access to growth capital would be severely limited (in comparison to being based in the US). To adapt for this major constraint, the company developed a strategy, *modus operandi*, where its focus would be on collaboration and out-licensing of the technology, with the company facilitating the development of new constructs, protecting intellectual property, and generating revenue from licensing and product supply (all underpinned by strong branding).

As can be seen in the following verbatim exerts from the 2001 and 2021 business plans (written by the author) the original *modus operandi* has remained essentially unchanged after 20 years.

> **2001 Business Plan**
>
> *Kiwi Ingenuity Limited ("KIWI") is a research company set up in 1996 which has developed and patented several innovative techniques enabling the control and manipulation of a natural phenomenon whereby molecules have the ability to insert in to cell membranes without causing damage to the cells. This insertion technology forms a platform technology known as "KODE".*

KIWI's mission is to be an innovative Research and Development company that develops time saving, risk reducing and affordable diagnostic and therapeutic solutions to the international biomedical/medical market.

KIWI will achieve this through:

- *The employment of world-class scientists.*
- *Ongoing in-depth analysis of marketplace needs and opportunities.*
- *Partnering arrangements with market leaders, which enhance the marketing opportunities for both parties' products.*

***The company focuses on discovering and patenting technology** in the area of glycobiology, and their commercial implementation. KIWI's underlying technology is based on the development of an innovative technique enabling the control and manipulation of a natural phenomenon whereby molecules have the ability to insert in to cell membranes without causing damage to cells. Since its inception KIWI has intensely researched this phenomenon and is now able to control the process and create novel molecules, which can be inserted into cell membranes. This insertion technology forms a platform technology known as "KODE" and represents a range of technologies based upon the insertion phenomenon.*

KIWI's revenue will be generated by:

- *licensing the technology*
- *commercial joint ventures to develop the patents*
- *sale of products/ingredients*
- *consultancy*

and 20 years later the 2021 Business Plan

*Kode™ Technology has been validated academically and commercially and is a platform technology that can produce countless unique "bio-shape-paints", with an almost limitless number of applications in life science. Because of this, our **commercialization strategy has focused on protecting intellectual property (IP) with patents and enabling others to utilize the technology to develop their own Kode™-based products.** Where possible, by having licensees fund their own R&D (either independently or through a subsidiary/SPV, or contract), product development, clinical testing, and commercialization, often with us having ongoing oversight, we are able to maximize our returns while minimizing our risk and expenditure.*

*The basic formula is the **more licensees** we have developing products in diverse areas, the **greater our chances of success**.*

Our mission is "to ignite a revolution with Kode™ surface modification nanotechnology by enabling our partners to enhance existing and create next generation products with a technology to shape life"

To achieve this KBL is focused on three core activities:

- *managing Kode™ Technology intellectual property;*
- *enabling licensees to take Kode™ products to market and;*
- *managing revenue, profit and ROI's.*

Over the 27 years since its inception in 1996 until 2023 Kode Biotech has changed from being predominantly an R&D company developing new intellectual property for out-licensing, to being an established company strategically managing a mature intellectual property portfolio, and with a focus on construct supply. All the same a substantial amount of product development still occurs, but this now is almost exclusively by collaborators and licensees.

Successes & failures

Like most companies Kode Biotech has had its fair share of successes and failures. Many of the successes have been described above, and it is worthy to restate that probably Kode Technology's greatest success is one which we did not invent the use for (Chapter 5. Kode Technology Application Themes: A potential immuno-oncotherapeutic). However, this was no dumb-luck event, as early on it was always believed that the greatest successes for Kode Technology would be developed by others, hence why the company early on developed a range of R&D constructs for the research community (i.e. to create serendipitous opportunities).

As for failures, without a doubt the biggest failure was attempting to develop over 5 years a fertility enhancement molecules product (Kode FEM), which came at a massive cost in both cash and resources. The original science (independently published) appeared good but the final outcome was to eventually prove that the original hypothesis was wrong. Despite being a failure from a product perspective, the driving forces over this time successfully resulted in the third generation Kode constructs. Other failures were more difficult to explain, for example the syphilis and SARS-Cov-2 antibody diagnostic assays could not gain any traction despite significant competitive advantages and being exceptional low cost assays. Similarly, although the blood grouping quality control, teaching and diagnostics products all worked well, they have only received regional (Australasia) and or localised update (rather than global). Herein sits a fundamental problem with the Kode Biotech business model, that is the licensing of technology B2B (business-to-business) results in the licensor company has very little effective control on marketing and product sales by the licensee.

Kode Technology methodology also has a shortcoming – that is it is too simple to use, and many times over the years the feedback has been "is it really that simple". Typically the methodology to modify the surface of a cell will require complex chemical and/or genetic manipulation procedures which involve many steps and can even take many months to achieve. In contrast, the simple incubation of a micromolar solution of FSL construct(s) with a cell, virus, liposome or any surface can achieve a stable (albeit temporary), quantitative and controllable labelling of that surface in less than 1 hour, without any further processing or washing required (see Kode Technology Illustrated Technology Manual, Appendix 2, Henry *et al., 2023*) does indeed seem to be "too good to be true", (despite being validated by numerous academic publications).

Future of Kode Biotech Limited

The future direction of Kode Biotech is about maintaining existing license relationships, focusing on maximizing the returns from existing composition-of-matter patents and maximizing revenue opportunities related to supply chain. The most exciting short-term opportunities are koded liposomes (Campbell, 2023), the veterinarian version of AGI-134 (a companion animal immuno-oncotherapeutic) and several cosmeceutical applications (under current evaluation).

Chapter 6. Concluding Remarks.

Kode Technology has been a lifetime journey for the author, seeded by concepts in the 1980's related to blood group glycolipids and how they behaved, through to final evolution as a technology capable of modifying any biological or non-biological surface with almost any small molecule. The question now needs to be asked, has it finished evolving? And the answer is probably yes, at least from the perspective that the base concepts relating the design of the constructs are developed. However there is still plenty of scope within the base technology to create more sophisticated spacers, including advancing development of spacers with on-demand cleavable or labile sequences, or those that can flip-flop the functional head across a membrane, TCMG spacers multi-functional heads and functionalised spacers (FfSL's). This list is not a wish list, but is an actual list of prototype FSL constructs that have already been built and tested, but have yet to be prioritised as commercial or research opportunities.

With respect to applications of the technology, as the functional head of an FSL can be almost anything, there is also an almost unlimited number of potential applications (some of which are alluded to as unpublished observations in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al*., 2023). Kode Technology has many opportunities to change paradigms in biological methodology, and perhaps a very salient example of this is that FSL-extracellular matrix constructs can allow for the growing of cells which correct apical-basal orientation and development of tight junctions onto non-porous surfaces (something that cannot be achieved by other means). There are many other examples of new techniques, including cell surface chemistry constructs, some of which have been disclosed in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al*., 2023).

There is no doubt that Kode Technology is the fastest method for biotinylation or glycosylation of any cell, surface or liposome. Also as can be seen in the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al*., 2023) its utility crosses into all aspects of biotechnology. However, Kode Technology is still probably one the best keep secrets in biological sciences/ research, despite being reviewed in a several of journal papers, (Henry *et al*., 2011a; Henry *et al*., 2011c; Henry & Bovin, 2018; Henry *et al*., 2018a; Henry, *2020;* Korchagina & Henry, 2015; Korchagina *et al*., 2012) and overviewed at a few conferences (Henry & Bovin, 2008; Henry *et al.,* 2011c; Henry *et al*., 2010a; Henry, 2010b; Korchagina *et al*., 2013). As stated above (Kode Technology Theme: Kode Biotech Limited - Successes & failures) its simplicity of use has been a negative factor in its uptake, but in reality the lack of substantial marketing budget by Kode Biotech Limited has also been a significant factor. This is changing and momentum is building, and it is hoped the writing of the Kode Technology Illustrated Technical Manual (Appendix 2; Henry *et al*., 2023) will facilitate uptake of the technology and deliver on the mission statement of Kode Biotech, which is

"to ignite a revolution with Kode™ surface modification nanotechnology by enabling our partners to enhance existing and create next generation products with a technology to shape life"

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To help identify those journal papers relating to the authors work, the first authors name and the authors name are highlighted in blue font. Those references in the text (e.g. Frame et al., 2007) and in this reference list where the first authors name is also underlined are available as online copies via links provided in Appendix 1. Postgraduate student work cited and primary supervised by the author have the student name indicated in blue font. Patents are only listed under their WO publication number, with those patents where the author is an inventor indicate in blue font. Each patent is hyperlinked to the World Intellectual Property Organisation (WIPO) IP Portal (WIPO link) where the abstract, inventors and other patent information are summarised. Additional independent references related to Kode Technology can be found in the reference list of the Kode Technology Illustrated Technical Manual, Appendix 2

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WO 2004/072306 Preparation of red blood cells with a modified level of blood group antigen expression and their use in quality control of blood typing reagents (WIPO link)

WO 2005/121322 Enzymatic modification of cell-surface H antigen by glycosyltransferases (WIPO link)

WO 2005/090368 Synthetic membrane anchors (WIPO link)

WO 2007/035116 Cell surface coating with hyaluronic acid oligomer derivative (WIPO link)

WO 2008/030115 Fluorescent cell markers (WIPO link)

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WO 2009/035347 Peptide-lipid constructs and their use in diagnostic and therapeutic applications (WIPO link)

WO 2009/048343 Functional lipid constructs (WIPO link)

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WO 2012/121610 In vivo methods of monitoring biodistribution (WIPO link)

WO 2012/118388 Peptide-Lipid constructs and their use in a FSL-function assay (WIPO link)

WO 2013/081471 Facile laboratory method for localising biomolecules to the surface of cells and viruses (WIPO link)

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Appendix 1: Published Kode Technology Journal Papers

Click on the link below in the index to view an online copy of the journal paper. Most articles are freely available, however if the article is behind a firewall and cannot be accessed via the bookmark provided (e.g. requires an institutional login), then please write to the author and request a copy (shenry@kodebiotech.com)

Patents are not included in this list but are instead listed under their WO publication number in the References with a hyperlink to the World Intellectual Property Organisation (WIPO) IP Portal (WIPO link) where the abstract, inventors and other patent information are summarised.

The extent of contribution of the author and collaborators has been documented and declared in Co-authorship Declarations relating to the journal articles listed below (and the eBook included in this thesis). This declaration document is available as a separate pdf document on request.

Link to article	**Reference title** (for full reference details see references)
Barr *et al.*, 2014	Mapping the fine specificity of ABO monoclonal reagents with A and B type-specific FSL constructs in kodecytes and inkjet printed on paper.
Barr *et al.*, 2015	Monoclonal anti-A activity against the FORS1 (Forssman) antigen.
Barr *et al.*, 2016	Biofunctionalizing nanofibres with carbohydrate blood group antigens.
Blake *et al.*, 2011 Video link	FSL Constructs: A Simple Method for Modifying Cell/Virion Surfaces with a Range of Biological Markers Without Affecting their Viability.
Frame *et al.*, 2007	Synthetic glycolipid modification of red blood cell membranes.
Georgakopoulos *et al.*, 2012	An improved Fc function assay utilising CMV antigen coated red blood cells generated with synthetic Function-Spacer-Lipid constructs.
Hadac *et al.*, 2011	Fluorescein and radiolabeled Function-Spacer-Lipid constructs allow for simple in vitro and in vivo bioimaging of enveloped virions.
Harrison *et al.*, 2010a	A synthetic globotriaosylceramide analogue inhibits HIV-1 infection in vitro by two mechanisms.
Harrison *et al.*, 2011	A novel VSV/HIV pseudotype approach for the study of HIV microbicides without requirement for level 3 biocontainment.
Heathcote *et al.*, 2010	Novel antibody screening cells, MUT+Mur kodecytes, created by attaching peptides onto erythrocytes.
Henry, 2009	Modification of red blood cells for laboratory quality control use.
Henry *et al.*, 2011a	Designing peptide-based FSL constructs to create Miltenberger kodecytes.
Henry *et al.*, 2012b	Modeling transfusion reactions with kodecytes and enabling ABO-incompatible transfusion with Function-Spacer-Lipid constructs.
Henry, 2014a	Magnetic bead coatings: Today and tomorrow. Chapter 5 Rapid biofunctionalization of magnetic beads with function-spacer-lipid constructs. SepMag eBook 2014 (http://sepmag.eu/free-guide-magnetic-bead-coatings)
Henry *et al.*, 2018a	Applications for kodecytes in immunohematology.
Henry *et al.*, 2018b	Rapid one-step biotinylation of biological or non-biological surfaces.
Henry & Bovin, 2018	Kode technology – a universal cell surface glycan modification technology.
Henry *et al.*, 2022	Continuous population surveillance of COVID-19 immunity can be provided by blood services at low cost using routine laboratory infrastructure.
Henry, 2020	Kodecytes: modifying the surface of red blood cells.
Hult *et al.*, 2012	Flow cytometry evaluation of red blood cells mimicking naturally-occurring ABO subgroups following modification with variable amounts of function-spacer-lipid A and B constructs.
Ilyushina *et al.*, 2014	Labeling of influenza viruses with synthetic fluorescent and biotin-labeled lipids.

Korchagina *et al.*, 2012	Toward creating cell membrane glycolandscapes with glycan lipid constructs.
Korchagina & Henry, 2015	Synthetic Glycolipid-like Constructs as Tools for Glycobiology Research, Diagnostics and as Potential Therapeutics.
Lan *et al.*, 2012 Video link	Fluorescent Function-Spacer-Lipid construct labelling allows for real-time in vivo imaging of cell migration and behaviour in zebrafish (Danio rerio)
Nagappan *et al.*, 2021	COVID-19 antibody screening with SARS-CoV-2 red cell kodecytes using routine serologic diagnostic platforms.
Oliver *et al.*, 2011a	Modeling transfusion reactions and predicting in vivo cell survival with kodecytes.
Oliver *et al.*, 2011b	In vivo neutralization of anti-A and successful transfusion of A antigen incompatible red cells in an animal model.
Perry & Henry, 2015	Training students in serologic reaction grading increased perceptions of self-efficacy and ability to recognise serologic reactions but decreased grading accuracy.
Perry *et al.*, 2016a	Antibody complement-mediated hemolytic studies with kodecytes reveal human complement utilized in the classical pathway is more stable than generally accepted.
Perry *et al.*, 2019	A standardized kodecyte method to quantify ABO antibodies in undiluted plasma of patients before ABO incompatible kidney transplantation.
Perry *et al.*, 2020	Incidence in plasma of low level antibodies against three xenotransplantation and immunotherapeutic glycan antigens.
Perry & Henry, 2022	Simulated red cell antibody identification training panels created using SARS-CoV-2 kodecytes and immune plasma.
Popova *et al.*, 2019	Synthesis of blood group Forssman pentasaccharide GalNAcα1-3GalNAcβ1-3Galα1-4Galβ1-4Glcβ–R.
Rapoport *et al.*, 2021	Localization of synthetic glycolipids in the cell and the dynamics of their insertion/loss.
Ryzhov *et al.*, 2016	Function-Spacer-Lipid constructs of Lewis and chimeric Lewis/ABH glycans. Synthesis and use in serological studies.
Ryzhov *et al.*, 2021	SARS-CoV-2 Peptide Bioconjugates Designed for Antibody Diagnostics.
Slivka *et al.*, 2022	Influence of the Lipid Moiety Structure on the Insertion/Release of Glycolipids in/from the Cell: A Study with Synthetic Analogs.
Srivastava *et al.*, 2021	COVID-19 antibody detection and assay performance using red cell agglutination.
Svensson *et al.*, 2013	Forssman expression on human erythrocytes: Biochemical and genetic evidence of a new histo-blood group system.
Tesfay *et al.*, 2013	PEGylation of vesicular stomatitis virus extends virus persistence in blood circulation of passively immunized mice.
Weinstock *et al.*, 2021	Erytra Blood Group Analyser and Kode Technology testing of SARS-CoV-2 antibodies among convalescent patients and vaccinated individuals.
Williams *et al.*, 2016a	Ultra-fast glyco-coating of non-biological surfaces.
Williams *et al.*, 2016b	Glycomapping the fine specificity of monoclonal and polyclonal Lewis antibodies with type-specific kodecytes and FSL constructs on paper.
Zalygin *et al.*, 2020	Structure of Supramers Formed by the Amphiphile Biotin-CMG-DOPE.

Appendix 2: The Kode Technology Illustrated Technical Manual

The Kode Technology Illustrated Technical Manual (Henry *et al*., 2023) is an eBook published in February 2023 and discusses all aspects Kode Technology including its scope, chemistry, features, methodology, applications, limitations and potential, and references this information from every known Kode Technology publication. A copy of this eBook is included in the following pages. However, the eBook is best viewed on a computer and a copy can be downloaded from

https://natlib.govt.nz/records/50606977