150 Years of Vaccine Science – Philip L. Felgner

More than 1000 years ago there was a procedure called variolation that exposed scratched skin of naïve individuals to pus from the sores of smallpox infected people and there was evidence and growing acceptance that variolation conferred protection from smallpox infection. In 1796 Jenner first showed in 23 humans (including his newborn son) that cowpox virus collected from the pus of infected cows could be used instead of human smallpox pus to confer protection against smallpox infection. Fifty years after Jenner's death (circa 1880), a network of 'Vaccine Farms' began to be established that could eventually provide enough cowpox virus to vaccinate the world and 100 years later we rid the world of smallpox.

150 years ago (1880-1910) Pasteur and Koch were busy establishing germ theory and identifying specific microorganisms responsible for infectious diseases and plagues. Jenner's smallpox vaccination then provided a roadmap for developing effective vaccines against other microorganisms. The immunological mechanisms responsible for effective vaccines were unclear and Paul Ehrlich discovered serum factors he called antibodies that interfere with microorganism proliferation. The vaccine science developed by Koch, Pasteur and Ehrlich in the late 1890s was applied by a leader in modern vaccine science, Maurice Hilleman. Between 1950-70 Hilleman led development of 12 vaccines at Merck that we still give to our children today.

By any reasonable standard, in the 21st century the process for manufacturing vaccines based on technology first developed by Koch and Pasteur in the 19th century is primitive. The first step is to propagate 100s millions of doses of infectious microorganisms and do this without infecting the people involved in the manufacturing. For example, still today most of the seasonal influenza vaccine is produced by propagating the predicted infectious seasonal influenza virus in chicken eggs in a process that takes 6 months. It takes one chicken egg to produce 1 vaccine dose against one influenza virus strain and four eggs for one dose of the quadrivalent influenza vaccine. For 200 million vaccine doses required to vaccinate the USA population every year, 800 million embryonated chicken eggs are required. One chicken produces 1 egg per day, so we need 10s million specific pathogen free chickens. Fortunately, the USA chicken egg industry produces 100 billion chicken eggs per year, so we have the capacity in the USA to produce 1 billion embryonated chicken eggs for influenza vaccine production every year.

Pharmaceutical product development and manufacturing is expensive and time consuming. A drug AZT developed in the 1960s for the treatment of HIV/AIDS received accelerated FDA approval in 1987. The drug developers worked fast and the time between the first demonstration that AZT was active against HIV in the laboratory and its approval was 25 months, the shortest period of drug development, until now.

The rapid pace of discovery, development, and FDA approval of the spectacular COVID mRNA/LNP vaccines in only ten months has taken vaccinologists, the scientific community, and the public by surprise. Within months after introduction and FDA approval of the vaccine, manufacturers Moderna and Pfizer produced and deployed billions of COVID vaccine doses. Then recently within months, they were able to deploy worldwide a modified version against newly emerging COVID variants. In addition to the spectacular efficacy of this modern vaccine technology the manufacturing of vaccines has finally moved from a 19th century technology into the 21st century.

Following introduction of the mRNA vaccine the public asked questions about safety of this new technology. The vaccine scientists often replied that the vaccine technology is not new and has been under development for 35 years. That answer prompted the next question, "Why did it take so long to make it available". These vaccines may seem to have suddenly emerged, but their advent is based on more than 35 years of scientific discovery, discourse and development, work from hundreds of scientists, numerous biotechnology companies and billions of public and private dollars invested enabling this effective response emerging from the scientific community so efficiently at this moment.

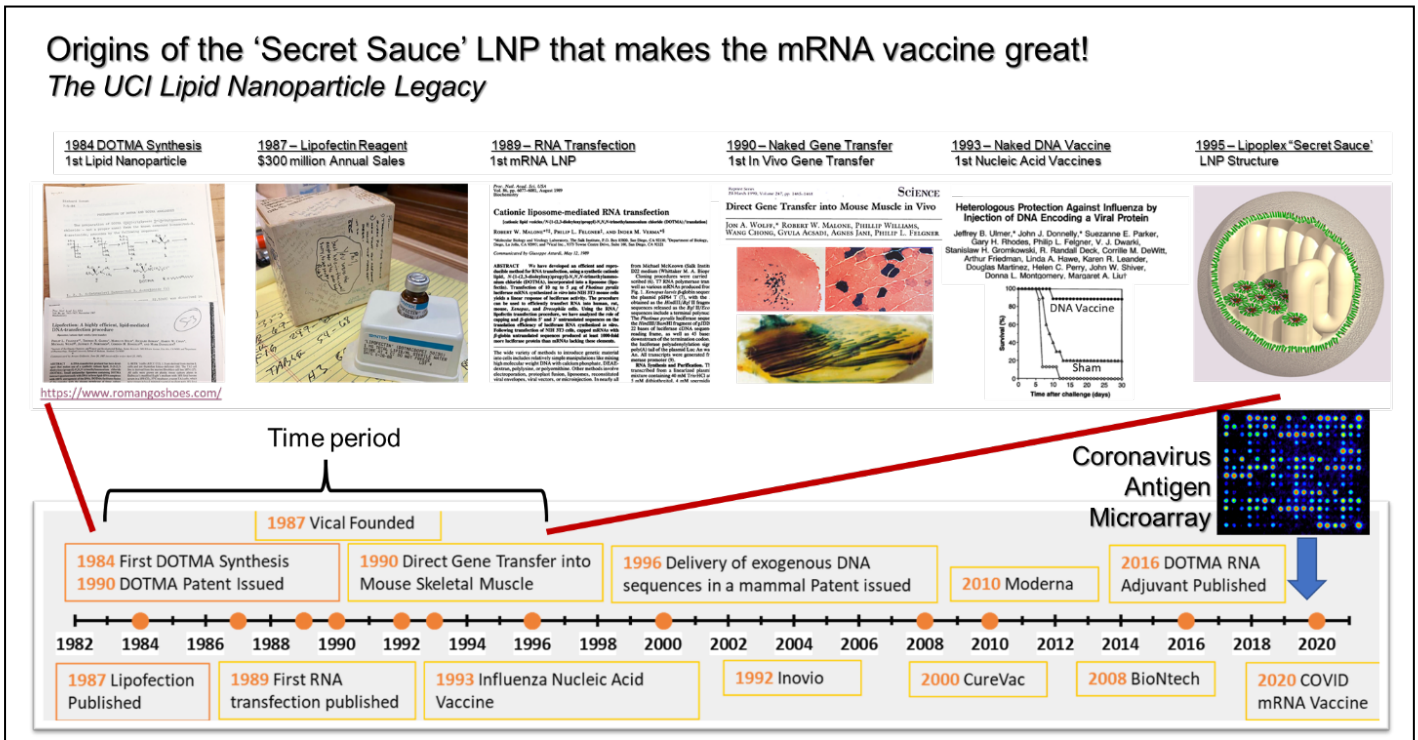
Origins of the mRNA lipid nanoparticle vaccine

Basic Lipid Nanoparticle (LNP) science began with the recognition of phospholipid vesicles called "liposomes" by Alec Bangham in 1965. Alec was a translationally oriented scientist who envisioned liposomes as 'magic bullets' that could deliver encapsulated drugs to the target cell. He attracted like-minded colleagues, students and post docs working to develop liposome drugs. The field attracted interest from investors in the 1980s and several liposomal drugs are approved.

I began my post-doctoral training in biophysical sciences at the University of Virginia in Tom Thompsons laboratory. Translational science was the farthest from Tom mind. He was mystified how people could come up with uses for this technology. He had a beautiful negatively stained electron micrograph of pure 70 nm diameter phospholipid vesicles next to his desk. He wanted to understand how the 4 nm thin bilayer membrane made up of lipid monomers is so remarkably stable. This membrane is necessary scaffold for life to exist so that individual cell remain discrete. He wanted to understand molecular dynamics of bilayer lipid membranes, how the molecules are organized, how the shape lipid molecules determine membrane stability, how strong is the bilayer membrane, and how mobile are the molecules. In the 1980s my basic science training converged with the nascent molecular biology, gene therapy, and immunology sciences and I made a series of discoveries that launched active fields of scientific research that did not exist before this work that

is rooted in the biophysics, structure and hydrodynamic properties of emulsions, micelles and bilayer membranes.

In 1984, there were no positively charged bilayer forming lipids available in nature to prepare positively charged lipid vesicles. At the time, we understood the spatial requirements of lipid monomers necessary to form macromolecular lipid bilayer structures and stable liposomes. In collaboration with chemists at Syntex Research Institute we synthesized and characterized a series of positively charged bilayer forming lipids. These positively charged liposomes interact with the negatively charged surface of cultured cells and they enter the cells, and they have may applications for drug delivery. Cationic liposomes also interact spontaneously with negatively charged nucleic acid and self-assemble into a new highly organized stable structure called 'lipoplex.' This discovery led to a series of 6 surprising results that set the foundation for my work on nucleic acid vaccines and summarized in the timeline below.



1. Mixing of positively charged liposomes with negatively charged nucleic acid was predicted to produce aggregation. Surprisingly, no change in turbidity was evident. Sucrose density gradient centrifugation was used to show that all of the input DNA floated to the top of the gradient along with the lipid, proving that they do interact as predicted. The lack of aggregation is a consequence of the lipid and DNA condensing to form a new stable structure called 'lipoplex' in which the fluid lipids change their packing to accommodate the nucleic acid. This synthetic self-assembled virus-like particle lacks an internal aqueous capture volume a characteristic of liposomes. Today we call these Lipid Nanoparticles.
2. We expected to add cell recognition ligands or antibodies to the lipoplex to achieve nucleic acid delivery to cells. Surprisingly, it worked right away without additional functionality. Encoded proteins were efficiently expressed using a simple transfection protocol. Simply mixing the cationic liposomes with DNA or mRNA, and adding the mixture to cultured cells resulted in expression of the encoded proteins.
3. It wasn't surprising that this convenient transfection reagent would be popular, but it is surprising how popular it became. Lipofectin and the similar reagent Lipofectamine sell $300 million/yr for Thermo Fisher.
4. I approached Syntex management to ask for approval to carry out an in vivo gene delivery study in mice using Lipofection technology. I was surprised when they declined, saying, "Gene Therapy is for the Year 2020".
5. I founded a start-up company with some faculty from UC San Diego to conduct the in vivo gene delivery study in mice and was surprised when it worked the first time. We could get reporter gene product levels in mouse muscle equivalent to that we get in cultured cells.
6. I tried to generate an immune response against HIV gp120 after intramuscular injection of DNA and mRNA encoding gp120 gene and was surprised when it worked the first time.

The progression of science is like a fabric - threads of knowledge woven together over time to improve our understanding and to benefit humanity.

In the mid-1980s, the topic of gene therapy involved cloning a gene of interest into recombinant virus vectors, repurposing the virus's infection and gene delivery machinery. Another branch of scientists envisioned encapsulating nucleic acid into liposomes to chemically construct a fully synthetic virus-like particle from the ground up for gene therapy. The liposome approach had a technical challenge because the long dimension of plasmid DNA is greater than the internal diameter of a liposome. Consequently, DNA encapsulation efficiency is extremely poor. I predicted that positively charged liposomes would interact with negatively charged DNA and agglutinate to capture 100% of the DNA into aggregates. Surprisingly, my first experiments mixing DNA and cationic liposomes produced no obvious change in the appearance of the solution, no visible evidence of aggregation of the two oppositely charged macromolecules.

To prove that DNA interacts with cationic liposomes, I could show that centrifuged free DNA goes to the bottom of a sucrose density gradient, but the cationic lipid DNA complexes float. We were able to show that by simple extemporaneous mixing DNA and cationic liposomes reorganized to form a different highly organized 100 nm virus-like

particle called ‘lipoplex’. My team hypothesized the lipoplex would be a versatile platform to optimize by rational design with ligands and receptors to improve gene delivery properties of these synthetic virus-like particles when we got another surprise. The lipoplexes already worked to deliver functional nucleic without any additional added features. Instead, optimization was accomplished by high throughput screening of different molecules and formulations rather than by rational design. The criteria controlling transfection efficiency were related to the lipoplex poised at the transition between the bilayer and the fusogenic hexagonal-II phases which could be empirically optimized using our high throughput transfection assay. We were struck by how straightforward it was to introduce functional genes into viable cells affecting the cellular phenotype – it was stunning to realize we had this convenient and effective tool to study the function of any protein in cells. Today lipofection is routine laboratory method used around the world to introduce functional mRNA or plasmid DNA into cultured cells, enabling advances in all areas of biological science research, and biotechnology. My groundbreaking research efforts in this space also resulted in a bustling $300 million worth of lipofection reagents sold worldwide every year, providing the global community with tools leading to lifesaving treatments for infectious diseases, cancer, and autoimmune disorders.

At Syntex Research Institute in Palo Alto where this discovery research was conducted, I approached leadership with a proposal to test whether the lipoplex that works so well in cultured cells would also work in vivo. They declined saying that “Gene Therapy is for the Year 2020”. This uncanny prediction resonates today since both mRNA vaccines were approved in the month of December 2020 – in the nick of time! Lacking support from Syntex, I was approached to join some UCSD faculty and started a company in San Diego called Vical. The investors agreed to support a ‘back burner’ gene therapy project to see whether lipofection could deliver genes in vivo. Like other things we did in this research area, it worked the first time, and my team was successful in showing mRNA and DNA delivery and expression in mice and rats.

Researchers working in gene therapy science noticed that the transgene expression measured after viral gene therapy was transient, which was eventually attributed to an immune response induced against the transgene product. While this observation was the bane of somatic gene therapy, it was a boon for nucleic acid vaccines when the intention is to induce a potent immune response against the gene product. A further advantage came out of new knowledge of two antigen processing pathways. Proteins that are transported into a cell are processed by the MHC Class 2 pathway, activate helper T cells and stimulate humoral immunity (antibodies) that can confer protection from infection. Proteins synthesized within a cell are processed by a separate MHC Class 1 pathway and stimulate CD8+ killer T cells. Thus, in addition to disease prevention, nucleic acid vaccination could potentially be used to treat preexisting chronic disease as AIDS and cancer.

My team’s first demonstration of nucleic acid immunization came in a collaboration with Chiron Pharmaceuticals which was developing a recombinant protein-based vaccine against HIV. They had an HIV gp120 plasmid DNA construct that expressed high levels of secreted gp120 in transfected mammalian CHO cells. We injected animals with this plasmid and demonstrated DNA vaccine induced antibodies against HIV gp120 Abs and cellular immune responses, and we were fortunate this breakthrough also worked the first time we tried. In partnership with Merck, we demonstrated that DNA vaccination using the sequence conserved nucleocapsid protein from influenza induces a potent cross strain protective immune response in mice. Merck's Maurice Hilleman, a leading American microbiologist who specialized in vaccinology and developed over 20 vaccines, said a major value of DNA immunization is that it can trigger immune responses against proteins without triggering one against the plasmid. Theoretically, this means the plasmid vector could repeatedly deliver DNA coding for different proteins and thus protect against a variety of diseases. "This is one of the most exciting things in modern vaccinology," said Hilleman. For the first time, we had a practical way to transduce cells and study the effect of gene on the phenotype of cells and present intracellular antigens to the immune system through the MHC Class 1 pathway.

As the next step in my research journey, two things became evident. First, we needed a way to select the best antigens to be used in vaccines, and secondly, genome sequencing methods were developing in the early 2000s that could help accomplish that. I moved from Vical to UCI to create a high throughput cloning & expression platform. In 20 years since then, my team cloned 70,000 individual genes using our high throughput PCR/recombination cloning method and express the proteins in a cell free system in vitro. We developed a protein microarray chip printing approach to print each of the individual protein from 45 bacteria, viruses, and parasites on the biodefense agents and emerging infectious disease agents list. The laboratory has printed 100,000 protein microarrays, probed 50,000 human serum specimens, discovered hundreds of immunodominant serodiagnostic vaccine antigens (Ags), and reported the results in 175 papers. This background fuels our current preclinical research on vaccines for *Coxiella burnetii* (Q Fever), influenza, leptospirosis, malaria and *Streptococcus pneumonia*.

Two years before the COVID-19 outbreak, my team constructed a protein microarray containing antigens from 6 respiratory viruses that cause common colds and flu, including a collection of common coronavirus antigens. The United States Defense Advanced Research Projects Agency, more commonly known as DARPA, supported our project to conduct serosurveillance in students residing in a large dormitory at the University of Maryland and passing respiratory

infections around to each other during flu season. We were prepared when the outbreak emerged in January 2020 to add a collection of SARS-CoV-2, SARS and MERS antigens to the array. Since then, we collected and probed more than 10,000 fingerstick blood specimens from Orange County residents and followed the gradual development of naturally acquired immunity in the Orange County community. We were prepared then with data from thousands pre-vaccination specimens to observe the spectacular efficacy of the mRNA vaccines in the same community. After an aggressive vaccination campaign at the UCI Medical Center, which began in December 2020 seroprevalence jump from 13% in December to 99% vaccine induced immunity in March 2021. After having planted the seeds and envisioning the benefits of nucleic acid immunization technology some 30 years earlier, it is thrilling to see it come to fruition now, and to participate in the analysis of its performance.

Personally, this decades-long vignette was a fascinating, and rewarding scientific journey. As an academic researcher, I encourage aspiring scientists to enter their own personal, discovery journey to benefit human society. My work supported the development of the first commercially available mRNA vaccines currently on the U.S. market, specifically the Pfizer-BioNTech and Moderna vaccines. My novel research efforts will inform the global response and management of SARS-CoV-2 and future outbreak preparation efforts. My lipofection DNA and RNA research efforts will continue informing vital treatment pathways for numerous cancers and related diseases. My vaccine antigen research efforts supported the Orange County region in the worst pandemic in a hundred years and will continue to pioneer and inform local and global clinical treatments across a multitude of disease scopes for years to come.