Viral Mimicking Nanoparticles as a Novel Vaccine Platform Against Bacterial Toxins

by

Zera Montemayor

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Doctoral Committee:

Professor Duxin Sun, Chair
Professor Wei Cheng
Professor James Moon
Professor Vincent B. Young

Zera Montemayor

zeram@umich.edu

ORCID iD: 0000-0001-5783-2973

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Dedication

For my grandma Josie-

You nurtured the spirited little girl I once was, urging my mom to preserve that spark within me. Your wisdom and guidance shaped me into the strong, independent, and fearless woman I am today. This dissertation is a testament to the spirit you protected and the strength you instilled in me. Thank you for believing in me and for teaching us girls the power of independence. Your influence has brought me to where I am today, and for that, I am eternally grateful.

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Abstract

This dissertation explores novel approaches to address significant challenges in infectious disease prevention through the development of a Virus Antigen-Mimicry nanoparticle (VAM) platform for enhanced vaccine delivery against bacterial toxins. We evaluated this platform against three bacterial pathogens whose pathology is primarily mediated by potent exotoxins: *Clostridioides difficile*, *Bacillus anthracis*, and *Clostridium botulinum*.

The VAM platform consists of ultrasmall gold nanoparticles (2-3 nm) conjugated to an iron-oxide core (~30 nm). Physical characterization via Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) confirmed that VAM particles fall within the desired size range (<200 nm), comparable to most viruses. Peptide conjugation assays demonstrated that VAM achieves high antigen surface density (10,000-40,000 antigens per μm^2), similar to viral surfaces. Importantly, the gold nanoparticles are clustered 5-10 nm apart, creating antigen patterns that mimic the key structural features of viruses that have evolved to optimize B cell receptor (BCR) crosslinking and activation.

In vitro studies demonstrated enhanced BCR engagement compared to controls lacking organized antigen clusters. This enhanced BCR crosslinking correlated with superior antibody production *in vivo*, where immunization studies in mice demonstrated that VAM induced antigen-specific IgG titers 3-14-fold higher than controls across all three bacterial toxin antigens. Unlike traditional toxoid vaccines or virus-like particles that require extensive engineering for each pathogen, VAM offers a simplified, adaptable approach requiring only cysteine-terminated peptides for conjugation to the platform.

We subsequently investigated oral vaccine delivery using this platform to stimulate mucosal immunity. We hypothesized that initiating the immune response at mucosal sites would lead to the production of secretory IgA antibodies, which parenteral

vaccines often fail to induce. These antibodies could provide protection at the primary sites of pathogen entry for many bacteria, including *C. difficile*. Initial attempts with direct oral gavage of aqueous VAM formulations failed to elicit detectable mucosal antibody responses, likely due to antigen degradation in the gastrointestinal tract.

To address this limitation, we developed enteric-coated gelatin capsules with four polymer coatings designed to protect lyophilized VAM from gastric degradation while enabling intestinal release. The lyophilization process was optimized using cryoprotectants (15% human serum albumin and 2% sucrose), which successfully prevented nanoparticle aggregation during the freeze-drying process. Despite these formulation advances, capsule delivery of lyophilized VAM failed to elicit detectable antigen-specific antibody responses in either serum or mucosal secretions.

These studies have addressed key challenges in developing versatile vaccine platforms against bacterial toxins while also revealing important limitations in our understanding of mucosal immunity stimulation. Our work successfully demonstrated that virus-mimicking structural organization of antigens can enhance systemic immune responses through improved BCR engagement and activation. However, the challenges encountered with mucosal immunization highlight critical knowledge gaps regarding oral vaccine formulation, antigen stability through the gastrointestinal tract, and optimal mucosal immune stimulation strategies. These findings underscore the complexity of developing comprehensive vaccine platforms and provide valuable direction for future investigations into alternative mucosal delivery routes and formulation strategies to achieve protective immunity at key pathogen entry sites.

Chapter 1 Vaccines for Infectious Disease

1.1 Introduction

Vaccination remains one of the best ways to protect ourselves against disease[1]. Not only can vaccination prevent people from getting ill, but it also prevents the spread of disease[2]. Since their discovery over 250 years ago, vaccinations have saved millions of lives[3]. Deadly diseases that once caused widespread seasonal outbreaks and regional epidemics have been significantly impacted by vaccination efforts. Smallpox has been globally eradicated, while polio has been eliminated from most countries worldwide[4]. For other diseases such as measles, diphtheria, and whooping cough, vaccination has dramatically reduced mortality rates among immunized individuals and lowered overall disease incidence in populations with high vaccination coverage[5]. Lastly, vaccines have an obvious health benefit, but they also have an economic benefit. In the US alone, there is a net economic benefit of $69 billion, due to vaccines reducing disease burden and preventing death[6].

1.1.1 Antigens

The main component of any vaccine is the antigen. An antigen is either the pathogen itself (live or dead) or parts of the pathogen. Many licensed vaccines utilize the whole pathogen[7]. Subunit vaccines, which use only specific parts of pathogens, have been established for decades and continue to be extensively developed due to their enhanced safety profiles[8,9]. Deciding which part (antigen) to use adds an additional layer of difficulty in designing vaccines. As an example, in the popular Covid-19 vaccines, the antigen was the spike protein of SARS-CoV-2[10].

The complexity of antigen selection varies significantly depending on the pathogen type. Viruses are relatively simple structures, typically consisting of nucleic acid encased within a protein shell, sometimes with an additional lipid envelope containing embedded proteins. This comparative simplicity often makes antigen

selection more straightforward for viral vaccines, though important challenges still exist[11,12]. In contrast, bacteria present considerably greater complexity due to their diverse cellular structures and virulence mechanisms[13].

Bacteria possess a multitude of potential antigenic target that could be incorporated into vaccine design[14,15]They have numerous surface proteins that aid in attachment to the host cell[16,17]. They can have a flagellum that provides motility allowing it to invade host cells[18,19]. Some bacteria are noninvasive but damage the host through potent toxins[20,21]. All these structures can be potential antigens. Additionally, some vaccines utilizing only one antigen lack efficacy, so designing a vaccine with multiple antigens is often necessary adding an extra layer of difficulty[22–24].

This thesis focuses specifically on designing vaccines against toxin-producing bacteria because toxins represent an ideal vaccine target for several reasons. First, bacterial toxins are often the primary virulence factors directly responsible for disease pathology, meaning neutralizing these toxins can prevent disease even if the bacteria remain present[25,26]. This is particularly important for pathogens like *Clostridioides difficile*, *Bacillus anthracis*, and *Clostridium botulinum*, where the clinical manifestations are predominantly caused by toxin activity rather than bacterial invasion or inflammation.

Second, toxins typically have conserved structures essential for their function, making them less prone to the antigenic variation that complicates vaccine development against other bacterial components[27,28]. The functional domains of bacterial toxins, especially those involved in catalytic activity, tend to be highly conserved across strains, providing a stable target for immune recognition.

Third, focusing on toxins allows for the development of non-living vaccines that eliminate safety concerns associated with whole-pathogen approaches while still targeting the disease-causing mechanism[29]. This approach has proven successful for vaccines against diphtheria and tetanus, which use modified toxins (toxoids) to induce protective immunity without the risks associated with live bacterial vaccines.

The specific mechanisms of bacterial toxins and their role in pathogenesis will therefore be discussed in detail in subsequent sections, providing the foundation for understanding the rational design of toxin-based vaccines.

1.1.2 Toxin Structure and Mechanism

There are many toxin-producing bacteria that are a public health issue. Licensed vaccines include the Diphtheria, Tetanus, and acellular Pertussis (DTaP) vaccine and the anthrax vaccine[30]. These vaccines are primarily composed of antigens that target the bacterial toxin. Bacterial toxins can be classified as exotoxins or endotoxins. Endotoxins, specifically lipopolysaccharide (LPS), are component of the outer membrane of Gram-negative bacteria and are released upon cell lysis or during bacterial replication[31]. Unlike the peptidoglycan cell wall, LPS forms the outer leaflet of the outer membrane and consists of lipid A (the toxic component), a core oligosaccharide, and an O-antigen[31]. Exotoxins, in contrast, are proteins actively secreted by both Gram-positive and Gram-negative bacteria and can be transported to various sites throughout the body[32]. This section will give a brief overview of general exotoxin structure and mechanism before discussing the specific toxins produced by the three bacteria that are the focus of this dissertation.

Many bacterial exotoxins exhibit an A-B structure, where functionally distinct components work cooperatively to deliver a toxic payload. In classical A-B toxins, such as cholera toxin, diphtheria toxin, and pertussis toxin, the B component (sometimes composed of multiple identical or non-identical subunits) is responsible for receptor binding and transport, while the A component possesses the catalytic activity[33]. For example, cholera toxin consists of a single A subunit surrounded by a pentameric ring of identical B subunits that bind to GM1 gangliosides on intestinal epithelial cells[34]. The B component (referring to either a single polypeptide or an oligomeric assembly of polypeptides) binds to specific host cell receptors[35]. This receptor specificity determines toxin tropism, targeting neurotoxins to neurons, enterotoxins to intestinal epithelial cells, and so forth[36].

The A component (typically a discrete polypeptide chain) contains the enzymatic domain that carries out the toxic activity. This domain binds to specific target proteins within the host cell, often inactivating or modifying them through various mechanisms including proteolysis, ADP-ribosylation, glycosylation, or dephosphorylation, which can ultimately lead to cell death[37–39]. These toxic activities are responsible for the pathology associated with bacterial infections[25].

Bacterial toxins can act either extracellularly (along the cell surface) or intracellularly. Intracellular-acting toxins require additional functional domains or mechanisms for host cell entry and processing. These typically include: (1) a receptor-binding domain within the B component, (2) a translocation domain that facilitates movement of the catalytic portion across cellular membranes, and (3) in many cases, an autoprocessing domain that mediates the release of the catalytic domain from the rest of the toxin structure once inside the cell[40,41]. The terminology of “subunit” generally refers to separate polypeptide chains, while “domain” describes a functionally distinct region within a single polypeptide[42].

The intracellular delivery of bacterial toxins typically occurs via one of two main endocytic pathways. In the “short pathway,” which is utilized by the three toxins examined in this dissertation (*C. difficile* toxins, anthrax toxins, and botulinum neurotoxins), the toxin first binds to its receptor on the cell surface, triggering endocytosis. Within the acidified environment of the endosome, the toxin undergoes conformational changes that enable the formation of a pore in the endosomal membrane, through which the catalytic domain translocates directly into the cytosol[43]. Understanding this pathway is relevant to vaccine development, as antibodies that interfere with receptor binding or conformational changes can neutralize toxin activity before cellular entry occurs[44–46]. The alternative “long pathway,” utilized by toxins such as cholera toxin and Shiga toxin, involves retrograde transport through the Golgi apparatus and endoplasmic reticulum before the catalytic domain enters the cytosol; however, this pathway is not utilized by the toxins that are the focus of this dissertation[47].

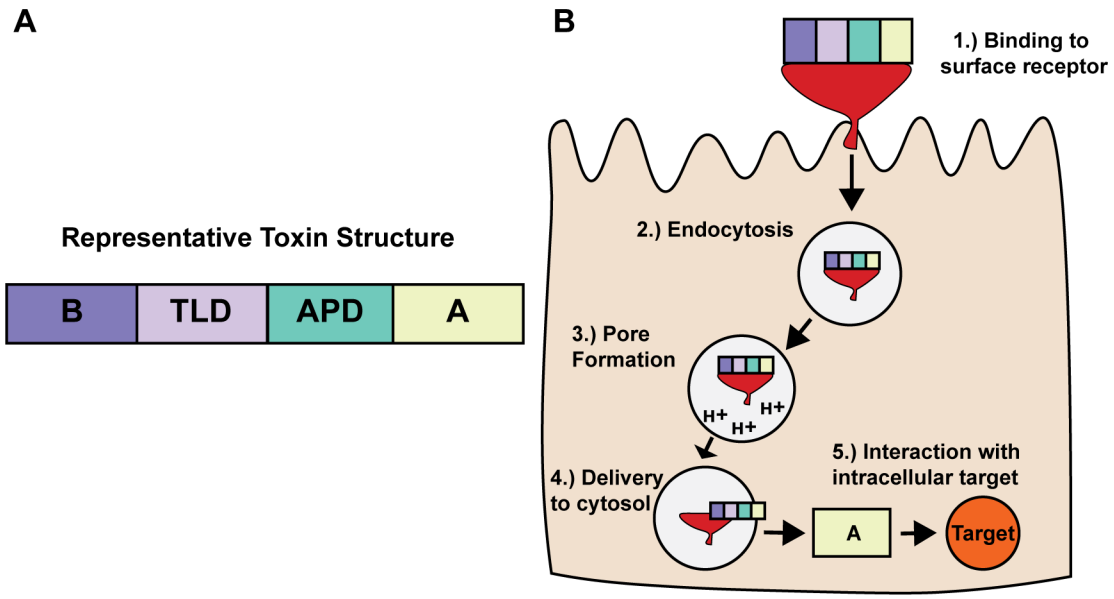


Figure1.1 Toxin structure and entry mechanism. (A) Representative toxin structure for intracellularly active toxins. (B) Short endocytosis pathway mechanism to deliver enzymatic domain to cytosol. B, binding subunit or binding domain; TLD, Translocation Domain; APD, Autoprocessing domain; A, A subunit or enzymatic domain.

1.1.3 Clostridioides difficile

Clostridioides difficile or *C. difficile* produces two major exotoxins: Toxin A (TcdA, 308 kDa) and Toxin B (TcdB, 270 kDa). These large single-chain protein share approximately 45% sequence homology, and a similar multi-domain structure comprised of four functionally distinct regions[48].

The C-terminal Receptor Binding Domain (RBD) mediates cellular entry through interaction with host cell receptors. This domain contains multiple Combined Repetitive Oligopeptides (CROPs) arranged in short and long repeats that form β-solenoid structures[49,50]. While structurally similar, TcdA and TcdB bind to different cellular receptors with varying affinities. TcdA primarily interacts with glycoprotein receptors containing carbohydrate structures such as Galα1-3Galβ1-4GlcNAc, while TcdB has been shown to bind multiple receptors including Poliovirus Receptor-Like 3 (PVRL3), Chondroitin Sulfate Proteoglycan 4 (CSPG4), and Frizzled proteins (FZDs)[51–53]. Additionally, there is evidence suggesting that both toxins can utilize non-CROP regions for receptor binding and cellular entry, highlighting the complexity of toxin-host interactions[53–55].

Adjacent to the RBD is the Delivery Domain, which undergoes a pH-dependent conformational change in the acidified endosome to form a pore in the endosomal

membrane. This pore facilitates the translocation of the enzymatic components into the host cell cytosol[56]. The Cysteine Protease Domain (CPD) functions as an autoprocessing domain that, upon binding to cytosolic inositol hexaphosphate (InsP6), catalyzes the proteolytic release of the N-terminal Glucosyltransferase Domain (GTD)[57].

Once released into the cytosol, the GTD exerts multiple pathogenic effects. Its primary activity is the mono-O-glucosylation of Rho family small GTPases (including RhoA, Rac1, and Cdc42), which prevents these molecular switches from interacting with their effectors[58]. This inactivation disrupts actin cytoskeleton dynamics, leading to cell rounding, loss of tight junction integrity, and increased epithelial permeability. However, the toxins' pathogenic effects extend beyond GTPase inactivation. TcdA and TcdB also induce the production of proinflammatory cytokines (including IL-1β, IL-8, and TNF-α) through activation of the inflammasome and NF-κB signaling pathways[59–61]. Furthermore, the toxins trigger multiple cell death pathways including apoptosis, necrosis, and pyknosis, depending on toxin concentration and cell type[62].

Collectively, these actions result in colonic epithelial damage, neutrophil infiltration, fluid secretion, and the inflammatory cascade characteristic of *C. difficile* infection (CDI)[63]. The resulting pseudomembranous colitis can range from mild diarrhea to fulminant colitis with potentially fatal complications[64].

1.1.4 Bacillus anthracis

Bacillus anthracis or *B. anthracis* produces three protein components that combine to form two distinct binary toxins: the Protective Antigen (PA, 83 kDa), the Edema Factor (EF, 89 kDa), and the Lethal Factor (LF, 90 kDa)[65]. Rather than functioning independently, these components work together in a precise sequence to deliver the enzymatic components (LF and EF) into host cells[66].

The intoxication process begins when PA binds to cell surface receptors—either tumor endothelium marker-8 (TEM8, also known as ANTXR1) or capillary morphogenesis protein-2 (CMG2, also known as ANTXR2)[67,68]. Following receptor binding, host cell proteases (primarily furin or furin-like proteases) cleave PA at a specific site, removing a 20 kDa N-terminal fragment (PA20) and leaving the receptor-bound 63 kDa fragment (PA63)[69]. This proteolytic activation enables PA63 monomers to self-assemble into a heptameric or octameric pre-pore structure[70].

The assembled pre-pore then serves as a docking platform for LF and/or EF, with each pre-pore capable of binding up to three or four molecules of LF/EF[71]. This toxin-receptor complex undergoes receptor-mediated endocytosis, and the acidic environment of the endosome triggers a conformational change in the PA pre-pore, causing it to insert into the endosomal membrane and form a functional transmembrane pore[72]. Through this pore, LF and EF are translocated into the cytosol where they exert their enzymatic activities[73].

Once in the cytosol, LF functions as a zinc-dependent metalloprotease that specifically cleaves and inactivates mitogen-activated protein kinase kinases (MAPKKs), disrupting critical cell signaling pathways and ultimately leading to cell death through mechanisms including apoptosis, pyroptosis, or necrosis depending on the cell type[74,75]. EF, on the other hand, is a calmodulin-dependent adenylate cyclase that dramatically increases intracellular cyclic AMP (cAMP) levels, leading to edema, impaired phagocyte function, and disruption of cellular water homeostasis[76,77].

The combination of PA with LF forms lethal toxin (LT), while PA combined with EF forms edema toxin (ET). Both toxins contribute to anthrax pathogenesis through complementary mechanisms that collectively undermine the host's immune defenses and promote bacterial survival and dissemination[78].

Anthrax infection can be acquired through multiple routes: cutaneous exposure (the most common form), ingestion, or inhalation of spores[79]. The inhalational form is the most fatal version of the disease, with mortality rates exceeding 80% even with appropriate antibiotic therapy if treatment is delayed[80]. Although anthrax is relatively rare in the United States, approximately 20,000-100,000 cases are reported globally each year, predominantly in rural areas with limited access to veterinary services[81]. Given its potential as a bioterrorism threat, as demonstrated by the 2001 anthrax letter attacks in the United States, developing effective vaccines against anthrax remains an important public health priority[82].

1.1.5 Clostridium botulinum

Clostridium botulinum or *C. botulinum* produces botulinum neurotoxin (BoNT), which exists in seven antigenically distinct serotypes designated A through G. Of these, serotypes A, B, E and F are primarily associated with human botulism, while serotypes

C and D typically affect animals, and serotype G is rarely associated with human disease[83].

Each botulinum neurotoxin is synthesized as a single polypeptide chain (~150 kDa) that is post-translationally cleaved to form a dichain molecule consisting of a heavy chain (~100 kDa) and a light chain (~50 kDa) linked by a disulfide bond[84]. The heavy chain contains two functionally distinct domains: a C-terminal binding domain (HC) that mediates specific binding to receptors on presynaptic nerve terminals, and an N-terminal translocation domain (HN) that facilitates the translocation of the light chain into the neuronal cytosol. The light chain functions as a zinc-dependent endopeptidase that selectively cleaves specific SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins [85]. This proteolytic activity prevents the fusion of synaptic vesicles with the presynaptic membrane, thereby blocking the release of acetylcholine at neuromuscular junctions and resulting in flaccid muscle paralysis[86].

Botulism can manifest in several forms depending on the route of exposure. Infant botulism is the most common form in the United States, occurring when infants ingest bacterial spores that colonize the intestine and produce toxin in vivo[87]. Other forms include foodborne botulism (from consuming preformed toxin in contaminated food), wound botulism (from toxin production in infected wounds), inhalational botulism (rare, but possible through aerosolized toxin), and iatrogenic botulism (from therapeutic misuse of botulinum toxin)[88,89]. Despite the different routes of exposure, all forms ultimately lead to the same clinical manifestation of descending flaccid paralysis.

Although naturally occurring botulism is relatively rare, *C. botulinum* neurotoxins are considered potential bioterrorism agents due to their extreme potency, with an estimated human lethal dose of approximately 1-2 ng/kg when delivered intravenously or by inhalation[90]. This extraordinary toxicity, combined with relative ease of production, has led to the classification of botulinum neurotoxins as Category A bioterrorism agents by the Centers for Disease Control and Prevention[91].

This brief overview highlights the complexity of bacterial pathogens. Though bacteria are small, unicellular organisms, they possess sophisticated mechanisms that can cause life-threatening diseases. The intricate nature of these toxins, their

complexity, and their severe health consequences underscores the importance of developing effective vaccines to prevent these infections.

1.2 Immune Responses to Vaccination

Vaccination can produce two types of immunity: humoral and cellular immunity. Humoral immunity, also known as antibody-mediated immunity, employs antibodies to defend against pathogens, while cellular immunity (or cell-mediated immunity), relies on T cells. The ideal immune response elicited by a vaccine depends largely on the pathogen's characteristics, its mode of infection, and its mechanisms of pathogenesis.

1.2.1 Humoral Immunity

Humoral immunity involves antibody production by B cells that can occur through various pathways[92,93]. While the classical pathway involves antigen-presenting cells (APCs) such as dendritic cells (DCs) that capture and process antigens, B cells can also directly recognize antigens without DC involvement[94,95]. B cells express membrane-bound immunoglobulin receptors (BCRs) that bind specific antigens, triggering activation signals[96].

This activation can occur in a T cell-dependent manner, where helper T cells (specifically CD4+ T follicular helper cells) provide essential signals through cytokines (IL-4, IL-21) and co-stimulatory molecules (CD40L), which are critical for robust antibody production, affinity maturation, and class switching. Without these T cell interactions, most B cell responses would be significantly impaired in both magnitude and quality. However, certain antigens like bacterial polysaccharides can activated B cells in a T cell-independent manner[97,98].

Following activation, B cells undergo clonal expansion and differentiate into antibody-secreting plasma cells. These plasma cells produce large quantities of antigen-specific antibodies that enter circulation, binding to their target antigens to neutralize pathogens, facilitate phagocytosis, or trigger complement activation[99]. Additionally, some activated B cells differentiate into memory B cells, which persist long after the initial exposure and enable rapid, high-affinity antibody production upon subsequent antigen encounters[100].

Most licensed vaccines rely on robust antibody production for protection particularly against extracellular pathogens and bacterial toxins[101]. Antibodies are especially effective against pathogens that cause disease through toxin production or extracellular replication, which aligns with the bacterial toxin focus of this thesis[102].

1.2.2 Cell-Mediated Immunity

Cell-mediated immunity operates primarily through direct cellular interaction rather than soluble antibodies. Naive T cells circulate through lymphoid tissues until they encounter their specific antigen presented by APCs[103].This interaction triggers T cell proliferation and differentiation into effector T cells that migrate to infection sites[104].

Cytotoxic T Lymphocytes (CTLs or CD8+ T cells) can directly eliminate infected cells by releasing cytotoxic granules containing perforin and granzymes[105]. Helper T cells (CD4+ T cells) serve multiple functions: they produce various cytokines that regulate immune responses, activate macrophages to enhance their microbicidal activity, provide essential signals for B cell activation and antibody production as described above, and support CD8+ T cell functions[106–108]. CD4+ T cells can differentiate into distinct subsets (Th1, Th2, Th17, Treg) based on the cytokine environment, each characterized by specific cytokine production profiles and immune functions[109].

1.2.3 Tailoring Immune Responses to Pathogen Type

The optimal type of immune response required for protection depends critically on the pathogen's characteristics[110]. Extracellular pathogens, including most bacteria and their toxins, are primarily controlled by antibodies that can neutralize toxins, prevent bacterial attachment, and facilitate phagocytosis[111]. For these threats, vaccines that induce strong humoral immunity are generally sufficient.

In contrast, intracellular pathogens such as viruses and certain bacteria (e.g., Mycobacterium tuberculosis, Listeria monocytogenes) that replicate within host cells are largely inaccessible to antibodies[112]. These pathogens require cell-mediated immunity, particularly CD8+ T cell responses that can recognize and eliminate infected cells.[113,114].

For complex pathogens with both extracellular and intracellular phases, or those that employ multiple evasion strategies, a balanced immune response involving both

humoral and cell-mediated components is often necessary[13]. This explains why next-generation vaccines against challenging pathogens increasingly aim to elicit both antibody and T cell responses[115,116].

The nature, magnitude, and longevity of immune responses depend on antigen presentation methods, highlighting the importance of vaccine platform selection in design strategies[117]. For vaccines targeting bacterial toxins, platforms that effectively present toxin antigens to B cells while also engaging helper T cells can generate optimal neutralizing antibody responses with robust immunological memory[118,119].

1.3 Classical Vaccines and Their Evolution

Classical vaccines typically involve utilizing whole pathogens that have been modified to prevent disease while still triggering a robust immune response. The history of vaccination dates back to 1796, when Dr. Edward Jenner demonstrated that inoculation with cowpox virus protected individuals against smallpox[120]. This groundbreaking work established the foundation for modern vaccinology.

1.3.1 Live Attenuated Vaccines (LAVs)

Live Attenuated Vaccines (LAVs) represent one of the earliest successful vaccine strategies. These vaccines contain living bacteria or viruses that have been attenuated (weakened) through laboratory processes, typically involving repeated passages in cell cultures or growth under suboptimal conditions[121,122].

LAVs closely mimic natural infections by presenting multiple antigens in their native conformation while simultaneously stimulating appropriate innate immune receptors, including toll-like receptors and NOD-like receptors[123]. This process induces local inflammatory responses at physiologically relevant sites, generating both robust humoral (antibody) and cell-mediated immune responses that closely resemble those seen in natural infection[124]. The comprehensive nature of this immune activation explains why LAVs typically provide strong and long-lasting protection[125].

However, this approach carries potential safety concerns. Attenuated pathogens can, in rare instances, revert to virulence through genetic mutations. A well-documented example is the oral polio vaccine (OPV), which has been associated with vaccine-derived poliovirus outbreaks in areas with low vaccination coverage[126]. Other examples

include the yellow fever vaccine, which has been associated with rare neurological complications[127].

Approved LAVs for viral infections include measles, mumps, and rubella (MMR) vaccine, varicella (chickenpox) vaccine, and rotavirus vaccines[128]. LAVs designed for bacterial infections include the tuberculosis (Bacille Calmette-Guérin, BCG) vaccine, oral typhoid vaccine, and certain cholera vaccines[129,130].

1.3.2 Inactivated Vaccines

Due to the safety concerns associated with LAVs, alternative vaccine technologies emerged, including inactivated vaccines. These vaccines contain pathogens that have been completely inactivated or killed, typically through chemical treatments (formaldehyde, β-propiolactone) or physical methods (heat, radiation)[131]. Since the pathogens are killed or inactivated the vaccines do not cause disease nor can the pathogen revert to its virulent form, generally enhancing safety compared to LAVs[132].

The inactivation process alters the pathogen's structure, modifying antigenic epitopes to varying degrees while preserving enough of the original structure to enable immune recognition[133]. Rather than remaining fully intact, the pathogens undergo significant conformational changes during inactivation, which influences both antigen presentation and the resulting immune response[134].

Contemporary research has revised earlier understandings of inactivated vaccines. While historically characterized as producing predominantly antibody-mediated responses, modern inactivation methods or adjuvanted inactivated vaccines can generate robust cell-mediated immunity as well[135,136]. The immune response to newer generations of inactivated vaccines can be substantial and durable, though multiple doses are still typically required to achieve optimal protection[137].

Examples of successful inactivated viral vaccines include those against poliomyelitis (IPV), hepatitis A, and certain influenza vaccines[132]. Bacterial inactivated vaccines include whole-cell pertussis vaccines, which have been largely replaced in developed countries by acellular pertussis vaccines due to reactogenicity concerns[138].

1.4 Subunit Vaccines Against Bacteria

Vaccines that utilize only parts of the pathogen instead of the whole organism can be broadly termed subunit vaccines. The evolution of pertussis vaccination illustrates both the benefits and challenges of transitioning from whole-cell to subunit approaches. The original whole-cell pertussis vaccine (wP), introduced in the 1940s, effectively reduced disease incidence but caused frequent fever and injection site reactions[139]. These adverse effects led to declining vaccination rates and a subsequent increase in whooping cough cases in the 1970s. After extensive research, a new generation of pertussis vaccines emerged in the late 1990s that used only specific bacterial components rather than the entire bacterium, termed acellular pertussis vaccines (aP).

The aP vaccines contain purified pertussis antigens, typically including inactivated pertussis toxin either alone or in combination with adhesion factors that help the bacterium attach to host cells[140]. These vaccines significantly reduced adverse reactions compared to wP vaccines, improving public acceptance and vaccination rates[29]. However, subsequent epidemiological studies have revealed that while aP vaccines effectively prevent severe disease, they may provide less robust and durable immunity than the whole-cell versions[141]. Several countries that switched from wP to aP vaccines have observed pertussis resurgence despite high vaccination coverage, suggesting differences in the quality and longevity of protection[138]. This experience with pertussis vaccines demonstrates how subunit approaches can improve safety profiles while potentially introducing new challenges regarding long-term efficacy, highlighting the complex balance vaccine developers must navigate.

1.4.1 Toxoid Vaccines

Toxoid based vaccines are made by inactivating the toxins produced by the bacteria, instead of using the whole bacteria. Heat and or chemicals are used to inactivate the native purified toxin; these are called toxoids. Vaccination with the toxoid produces anti-toxin antibodies, which can then bind to the toxin and neutralize it. These typically do not provide long term immunity, so booster doses are needed[142,143]. Examples of these vaccines are the tetanus and diphtheria vaccines[144].

1.4.2 Polysaccharide Vaccines

Historically, the first generation of vaccines against encapsulated bacteria utilized pure bacterial polysaccharide coats as antigens. These polysaccharide capsules were targeted because they represent major virulence factors that help bacteria evade the immune system[145]. Examples of these early vaccines included the first-generation products against *Haemophilus, Streptococcus, and Meningococcu*s vaccines developed in the 1970s-1980s[146]. These pure polysaccharide vaccines demonstrated significant limitations in clinical use[147–149]. While they could activate B cells through T-cell independent mechanisms and produce some antibodies, they failed to effectively stimulate T cell responses, resulting in poor immunological memory and limited protection[150]. They proved particularly ineffective in children under two years of age, who cannot mount adequate T-cell independent immune responses[146]. These limitations led to the discontinuation of most pure polysaccharide vaccines, which have since been replaced by conjugate vaccines that link the polysaccharide to protein carriers (discussed below)[151].

1.5 Enhancing the Immune Response of Vaccines

The historical experience with pure polysaccharide vaccines highlighted the need for improved approaches to subunit vaccine design, spurring the development of several strategies to enhance immunogenicity.

1.5.1 Adjuvants

Aside from the antigen, an adjuvant is a major component of most vaccines. Plainly, an adjuvant is a substance added to a vaccine that increases the immune response to an antigen. Adjuvants can be classified as immunostimulants or delivery systems. Immunostimulants act like pathogen-associated molecular patterns (PAMPS) or damage associated molecular patterns (DAMPS) and interact with pathogen recognition receptors (PRRs) on APCs to initiate the innate immune pathways[152]. This causes activation and maturation of APCs, which enhances their antigen presentation and expression of high levels of costimulatory signals and cytokines[153,154]. All of which allows for an enhanced adaptive immune response. Delivery systems do not directly interact with the immune system but are a vehicle for the antigens providing controlled

delivery to the target cell. The main function of delivery systems is to increase antigen uptake and presentation by APCs[155].

For 70 years, the only approved adjuvant was aluminum salts[156]. This remains the most widely used adjuvant in licensed vaccine and is effective at enhancing antibody responses[157]. Aluminum adjuvants primarily promote Th2-biased immune response, while having more limited effects on Th1-tpe immunity and cell mediated responses[158]. These adjuvants work through multiple mechanisms: they function as delivery systems by adsorbing antigens, which increases their retention at injection sites and enables more efficient uptake by antigen-presenting cells[159]. Additionally, aluminum adjuvants can trigger the release of damage-associated molecular patterns (DAMPs) and create a local inflammatory environment that promotes immune cell recruitment and activation[160]. Approved vaccines that contain aluminum adjuvants include: diphtheria, tetanus, meningitis, and hepatitis B[161]. In recent years, a growing number of vaccine platforms have been developed that rely on specialized delivery vehicles to target antigens to specific immune cells and tissues, expanding beyond the capabilities of traditional adjuvants.

1.5.2 Optimal Delivery

Delivery systems have significantly influenced vaccine research by addressing key challenges in generating robust immune responses. Effective vaccines typically need to stimulate both humoral immunity (antibody production) and cell mediated immunity, with the relative importance of each depending on the pathogen[133,162]. Lymph nodes serve as critical sites for both B and T cell activation, making efficient delivery of vaccine components to these anatomical locations essential for optimal immune responses[163,164].

Antigens can reach lymph nodes through multiple pathways. Some antigens may be captured by resident or migratory antigen-presenting cells (APCs) at injection sites, while others may drain directly to lymph nodes through lymphatic vessels[165]. However, passive drainage is often inefficient, with significant amounts of free antigen failing to reach lymphatic tissues, becoming diluted in circulation, or being rapidly degraded[166]. This inefficiency frequently necessitates higher antigen doses to achieve protective immunity[167].

1.6 Vaccine Delivery Platforms

Most vaccines are administered subcutaneously (SC) or intramuscularly (IM) in the upper arm, sites chosen for practical administration rather than optimal lymphatic drainage. While alternative injection sites closer to lymph node clusters could theoretically improve delivery, such approaches face practical and patient acceptance challenges[168]. Consequently, formulating vaccines with specialized delivery systems represents a more feasible strategy to enhance lymph node targeting, antigen stability, and presentation to immune cells[169]. These delivery technologies can dramatically improve vaccine efficacy while reducing the required antigen dose, making them central to modern vaccine development[170].

1.6.1 Messenger RNA (mRNA) Vaccines

mRNA vaccines are one of the newest vaccine platforms. They use mRNA to teach the immune system how to make a specific protein from that pathogen. The mRNA is then encapsulated by a lipid nanoparticle (LNP). This is necessary because mRNA antigens are susceptible to degradation by RNA enzymes. Encapsulation provides protection and ensures delivery of the antigen until it reaches the target cell[171]. These vaccines cannot cause disease. The mRNA vaccine functions as both an immunogen and adjuvant, thus producing a robust immune response[172]. Examples of mRNA vaccines include the Pfizer and Moderna Covid-19 vaccines[173].

1.6.2 Conjugate Protein Vaccines

Conjugate vaccines are made by conjugating a polysaccharide antigen to a carrier protein. Two common types of carrier proteins used in licensed vaccines are cross-reacting material (CRM) of diphtheria toxin and tetanus toxoid (TT)[174]. These proteins are inherently immunogenic, which improves immune recognition to the polysaccharide[175]. This vaccine induces a strong B and T immune response, which is characterized by a long-term memory response and high titer of antibodies, unlike the polysaccharide alone[176]. Examples of conjugate vaccines include the *Haemophilus influenzae type b* (Hib) and *Streptococcus pneumoniae* vaccines[177,178]. These vaccines demonstrate that we can design a platform to increase immune recognition of our antigen.

1.7 Concept of Viral Mimicry

To make an effective vaccine, the most important is ensuring optimal antigen presentation to the immune system. This is a downside of subunit vaccines, which alone typically aren't immunogenic. Unless these vaccines are formulated with a carrier, combined with an adjuvant, or a combination of the two, they won't evoke a strong immune response. Since, the most effective vaccines mimic natural infection, we should aim to develop vaccine platforms that incorporate key structural features from natural pathogens.

Studying viruses provides valuable design principles for vaccine development. Viruses are highly immunogenic dure to several key features. Their size in the 20-200 nm range allows for efficient trafficking to lymph nodes[179]. The surface of the virus is lined with repetitive antigens spaced 5-10 nm apart, a spatial arrangement that facilitates optimal B cell Receptor (BCR) engagement through multivalent interactions[180,181]. This specific spacing aligns with what has been termed the "density code" for BCR activation[182]. This spatial organization enables BCR clustering, which enhances downstream signaling cascades and leads to robust B cell activation, triggering immune responses that produce high titers of class-switched antibodies[183]. Viruses also stimulate T cell immunity through efficient antigen processing and presentation pathways[184]. A vaccine platform that incorporates these structural principles would be capable of producing comprehensive B and T cell immune responses.

1.7.1 Viral Vector Vaccines

Viral vector vaccines utilize harmless viruses to deliver genes encoding antigens from pathogens. Once delivered to host cells, these genes are transcribed and translated by the cell's machinery to produce the pathogen's antigenic proteins. The immune system then recognizes these expressed proteins and mounts an immune response against them[185]. These vaccines produce strong $CD4^+$ T and $CD8^+$ T cell responses and high amounts of antigen-specific antibodies[186]. This strong immune response can be produced with only one dose and without an adjuvant[185]. The most commonly utilized vector class are adenoviruses. The Ebola virus vaccine and the

Covid-19 vaccine from AstraZeneca and Johnson & Johnson utilize viral vector technology, respectively[187].

1.7.2 Virus-Like Particles (VLPs)

Virus Like Particles or VLPS are self-assembling proteins that mimic the structural features of viruses but lack the genetic material that causes infection. These vaccines mainly produce antibody-mediated immunity[188]. Since VLPs lack the viral genetic material, they are considerably safer than LAVs, or inactivated vaccines. An example of a currently approved VLPs is the human papillomavirus vaccine[189]. While these platforms that utilize viruses or their features have many benefits, there are also challenges which limit their adoption.

1.7.3 The Challenges of Viral Vector and VLP Platforms

An ideal vaccine platform should be universal, meaning we shouldn't have to extensively reformulate it for use against additional pathogens. However, VLPs and viral vector vaccines present significant manufacturing and reformulation challenges. Both systems require generation utilizing specialized cell cultures with optimized growth conditions and expression systems[188,190]. The reformulation process can be particularly complex. Incorporating new antigens often requires complete re-engineering of the expression systems, extensive sequence optimization, and sometimes fundamental redesign of the particle or vector itself[191]. Each new target antigen may require unique modification to maintain proper folding, orientation, and accessibility while preserving the structural integrity of the carrier[192].

Both platforms need to be extensively purified, which could involve multiple chromatography steps, ultracentrifugation and filtration processes to remove host cell proteins, nucleic acids, and other contaiminiants[193,194]. These purification requirements must often be re-optimized for each new construct, adding to development timelines. Different VLPs might need to be expressed in alternative host systems (bacterial, insect, mammalian, or plant cells) further complicating scalability and standardization[195].

For VLPs specifically, not every epitope can be successfully incorporated into the structure. Insertions at certain sites may disrupt critical protein-protein interaction necessary for particle assembly[193]. This disruption can potentially compromise structural

integrity and eliminate the benefits of viral mimicry that make VLPs attractive vaccine platforms[193]. Furthermore, even when epitopes are successfully incorporated, they may experience conformational changes that reduce immunogenicity or lead to the production of non-neutralizing antibodies[196].

The immune system strongly recognizes viral vector vaccines and VLPs as foreign, which drives their potent immunogenicity but also creates a significant limitation. These delivery systems can suffer from anti-carrier immunity[197]. This occurs when the immune system produces robust antibodies against both the intended antigen and the carrier itself. The resulting anti-carrier antibodies can neutralize subsequent doses of the same platform, significantly reducing or even preventing immune responses to new antigens delivered using the same carrier[198]. This phenomenon is especially true with adenovirus viral vector vaccines. Almost half of the United States population has preexisting immunity to adenovirus serotype 5 for natural exposure[199]. These individuals maintain circulating antibodies that can substantially compromise vaccine efficacy, limiting these platforms' utility for multiple pathogen or booster immunizations[200]. We will introduce our vaccine platform which captures many benefits of these aforementioned platforms while avoiding many of their limitation through ha fundamentally different design approach.

1.8 Hypothesis

In part one of this thesis, we discussed the two studies of our novel Viral Antigen Mimicry nanoparticle (VAM), which has the benefits of viral mimicry but lack the formulation issues of similar platforms. The general purpose of this thesis is to make vaccines against three toxin producing bacteria: *Clostridioides difficile, Bacillus anthracis,* and *Clostridium botulinum*. For *B. anthracis* and *C. botulinum,* which cause multiple forms of disease, we strategically selected specific disease manifestations to target. Our *B. anthracis* vaccine aims to prevent inhalation anthrax due to its severity and potential bioterrorism risk[80].For *C. botulinum*, we focused on preventing food-borne botulism for two key reasons. First, since our vaccine targets the botulism toxin rather than the spore, it has the highest probability of success against a toxin-mediated pathology[88]. Second, food-borne botulism represents one of the most common forms of human botulism, indicating a clear clinical need for preventative measures[88].

VAM demonstrates enhanced safety compared to traditional vaccines against these bacteria, as our platform does not utilize any live virus or toxin, only peptide antigens. This approach significantly reduces potential risks[201,202]. Furthermore, the platform's versatility is evident in our ability to develop three distinct vaccines by simply exchanging peptides without requiring extensive formulation modifications.

1.9 Aims

For this thesis, we established three primary aims for our VAM platform investigations:

Aim 1 (Chapter 2): To develop and characterize a Virus Antigen Mimicry nanoparticle platform with structural features that enhance antibody production against bacterial toxin antigens. We hypothesized that nanoparticles engineered with virus-inspired structural features (size <200nm, high density of surface antigens, and 5-10nm antigen clusters) would significantly enhance BCR engagement and antibody production compared to nanoparticles lacking these specific structural arrangements.

Aim 2 (Chapter 3): To evaluate whether oral delivery of VAM via enteric-coated capsules could effectively stimulate mucosal immunity. We hypothesized that protecting VAM from gastric degradation would enable the induction of antigen-specific mucosal IgA antibodies, which are critical for immunity at mucosal surfaces but difficult to achieve with parenteral vaccination.

Aim 3 (Part 2): To evaluate the clinical value of FDA-approved cancer drugs by quantifying their impact on patient outcomes. We hypothesized that despite high costs and regulatory approval, many recent cancer therapeutics provide only modest survival benefits. Through systematic analysis of clinical trial data, we aimed to determine the magnitude of Overall Survival (OS) and Progression-Free Survival (PFS) improvements associated with these treatments to inform evidence-based clinical and policy decisions.

These aims guided our experimental approaches and analyses throughout the thesis, with detailed methodologies and results presented in the subsequent chapters.

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Chapter 2 A Versatile Virus-Like Nanoparticle Platform for Enhanced Antibody Production Against Diverse Bacteria Pathogens

2.1 Abstract

Bacterial infections remain a significant global health challenge, causing over 7 million deaths annually. This study introduces a novel Virus Antigen-Mimicry nanoparticle (VAM) platform that enhances antibody production against bacterial toxins by incorporating key structural features inspired by viral organization. The VAM platform consists of ultrasmall gold nanoparticles (2-3 nm) conjugated to an iron-oxide core (~30 nm), creating antigen clusters spaced 5-10 nm apart, a spatial arrangement that facilitates optimal B Cell Receptor (BCR) engagement through multivalent interactions and receptor clustering.

We evaluated this platform against three significant bacterial pathogens: *Clostridioides difficile, Bacillus anthracis*, and *Clostridium botulinum*, focusing on their respective toxin antigens. In vitro, VAM demonstrated enhanced BCR engagement compared to control nanoparticles lacking the clustered antigen display. Mice immunization studies showed that VAM induced antigen-specific IgG titers 3-14-fold higher than controls, with similar trends observed in IgG1 and IgG2b isotypes. The platform demonstrated consistent performance across all three bacterial antigens, highlighting its versatility.

Unlike traditional toxoid vaccines or virus-like particles that require extensive reformulation for additional pathogens, VAM offers a simplified approach requiring only cysteine-terminated peptides for conjugation. This versatile platform addresses key limitations in current bacterial vaccine development through its optimized antigen presentation architecture and represents a promising strategy for preventing bacterial infections through effective vaccination, potentially reducing the global burden of bacterial disease.

2.2 Introduction

Vaccines remain one of the most important tools for preventing infectious disease. The World Health Organization led a study that estimated in the past 50 years over 150 million lives have been saved due to global immunization efforts[1]. In the U.S, we have vaccines that prevent more than 30 disease and infection, a majority of these are directed against viruses[2,3]. Vaccinating against bacteria has some challenges. Bacteria are generally more complex than viruses. They have a wide range of antigens, which antigens are immunogenic, and long lasting remains to be seen[4]. This study focuses on developing a vaccine against three bacterial pathogens: *Clostridioides difficile, Bacillus anthracis*, and *Clostridium botulinum*.

C. difficile is responsible for almost 500,000 infections every year in the U.S and is one of the major causes of nosocomial diarrhea[5]. This spore- forming bacterium also causes colitis and in rare cases death. The spore is transmitted via the fecal-oral route[6]. Spores are resistant to UV, desiccation, heat, and many alcohol-based cleansers, which aids its resilience[7]. The first line of treatment for *C. difficile* is antibiotic therapy[8]. However, antibiotics are one of the major risk factors for getting this infection[9].

B. anthracis (anthrax) is rare but serious infection. It is also a spore forming bacterium. There are multiple ways anthrax infections can be transmitted either cutaneous, ingestion, or inhalation. Cutaneous anthrax is the most common form and causes a painless sore on the skin, with appropriate treatment risk of death is very low[10]. Inhalation anthrax is the most severe form, even with treatment it is often fatal[11]. Anthrax is bioterrorism threat, caused by terrorist intentionally releasing spores into the environment.

Lastly, *C. botulinum* (botulism) is another rare but serious infection. Similarly to Anthrax, *C. botulinum* is transmitted via spores and can be acquired in various routes: iatrogenic, food borne, inhalation, wound, and infant[12]. Infant botulism is the most common form, with honey contaminated with spores being the most common source. With treatment infant botulism is rarely fatal (< 2%)[13]. All forms of botulism lead to muscle paralysis in the face and then moving towards the limbs[14]. Like Anthrax, botulism could be a bioterrorism threat[15].

All three bacteria produce toxins that are responsible for the associated pathology, rather than direct bacterial invasion itself[16]. These toxins represent strategic vaccine targets because they are often the primary virulence factors directly responsible for disease symptoms. Immunity against these toxins can prevent the most severe clinical manifestations even if bacterial colonization occurs[17]. A common vaccine technology towards these toxin-producing bacteria is a toxoid vaccine[18]. Toxoid vaccines are created by applying heat and/or chemicals to bacterial toxins, reducing their toxicity while retaining their ability to trigger an immune response. Pfizer and Sanofi both had *C. difficile* toxoid vaccine candidates make it to phase three trials but failed due to lack of efficacy[19,20]. There was an approved toxoid vaccine against botulism, but this was discontinued due to declining immunogenicity[21]. There is an FDA approved anthrax vaccine (Anthrax Adsorbed) licensed for pre and post exposure. It is a recombinant protein vaccine composed mainly of the protective antigen of the anthrax toxin with low amounts of the edema factor and lethal factor[22]. This vaccine is adjuvanted with aluminum hydroxide. The primary series requires four doses plus two additional boosters, and yearly boosters are necessary to maintain protection[23]. Designing a vaccine against these toxins creates some challenges.

The first issue we need to address is toxicity. While toxoids themselves aren't toxic, someone in the manufacturing process still has culture the deadly pathogen and isolate the toxins[24]. While the anthrax vaccine avoids this issue, all three vaccines use the toxin as the sole antigen, which introduces a second problem.

Toxin-based vaccines typically induce antitoxin antibodies as a means of protection against the pathogen. However, toxins exhibit strain variability, which may result in antibodies that fail to recognize and neutralize different toxin variants[25,26]. Additionally, while antitoxin antibodies provide valuable protection against disease symptoms, they generally do not prevent bacterial colonization or transmission, a recognized limitation in controlling overall disease spread. This explains the partial efficacy seen in the Pfizer and Sanofi clinical trials: high antitoxin antibodies titers provided some protection by neutralizing toxins and limiting severe disease but couldn't prevent initial infection[27]. Our approach focuses on toxin epitopes for several strategic reasons: they represent well-characterized targets with proven relevance to

pathogenesis, they contain conserved regions essential for toxin function that can be specifically targeted, and our VAM platform's enhanced immunogenicity may overcome the limitations of previous toxoid approaches by generating higher quality, more functional antibody responses[24,28,29]. While we recognize that complete disease control would ultimately benefit from vaccines that also prevent colonization, our current work focuses on demonstrating VAM's capacity to generate robust antitoxin responses as a crucial first step. Future iterations could incorporate additional bacterial surface antigens to create more comprehensive vaccines addressing both disease symptoms and transmission[30,31]. The limitations of current approaches highlight the need for more effective vaccine strategies against toxin-producing bacteria, which our VAM platform aims to address.

The first step to designing an effective vaccine against these bacteria is optimizing antibody production, as antibodies contribute to protection when part of a comprehensive immune response[32,33]. To promote robust antibody responses, a vaccine must effectively engage B cells through optimal antigen presentation. Recent research has revealed that antigen spatial organization and density on vaccine carriers fundamentally influence B cell activation through what has been termed the "density code"[34]. This code refers to how the precise spacing and density of antigens determine the threshold for BCR activation. When antigens are presented at appropriate densities, they can efficiently trigger BCR clustering, which enhances downstream signaling cascades and B cell activation. High-density, multivalent antigen display enables multiple low-affinity interactions to collectively strengthen avidity, mimicking the repetitive epitope patterns found on microbial surfaces. Consequently, vaccines consisting of soluble antigens simply mixed with adjuvants often generate suboptimal B cell responses due to their inability to present antigens in the proper spatial arrangement[35]. Similarly, vaccines using nanoparticles that encapsulate antigens rather than displaying them on the surface at appropriate densities fail to engage BCRs effectively[36]. While the strategic manipulation of antigen density is crucial for effective BCR engagement, this optimized presentation can only exert its immunological effects if the vaccine successfully reaches the anatomical sites where B cell activation predominantly occurs. Therefore, equally important to antigen density is ensuring that

the vaccine efficiently traffics to secondary lymphoid organs, particularly the lymph nodes[37].

The primary site where adaptive immunity occurs is lymph nodes[38]. Delivering antigen to the lymph nodes is another important feature for antibody production and vaccine design. However, peptides and small molecule antigens often struggle with poor lymph node delivery and low immunogenicity, limiting their ability to induce antibodies[39,40]. This is why delivery vehicles are typically necessary for robust and class-switched antibody production. These limitations highlight the need for innovative vaccine designs that can overcome these challenges and induce a potent and lasting immune response against bacterial pathogens.

To address these challenges in vaccine design, we can draw inspiration from viruses, which effectively traffic to lymph nodes, optimally engage BCRs through spatial arrangement, and potently activate the immune system[41]. Viruses typically range from 20-200 nm in size and feature antigen clusters spaced 5-10 nm apart, allowing for high antigen density on their surface[42]. This specific spacing is particularly effective because it aligns with the “density code” requirements for BCR clustering and activation. Rather than depending on individual high affinity interactions, this spatial arrangement facilitates multivalent engagements of BCRs enabling the crosslinking necessary for initiating signaling cascades. While using actual viruses as delivery vehicles has been explored, they pose safety risks and/or lack scalability[43,44]. Therefore, mimicking the advantageous physical properties of viruses (size and precise antigen spacing) presents a promising strategy for developing safer, more effective vaccines that can induce robust B cell responses.

Virus-like nanoparticles (VLPs) offer a promising alternative. These structures, composed of viral structural (capsid) proteins that self-assemble, mimic viruses while lacking infectious material, making them considerably safer[45]. VLPs maintain the beneficial features of viruses for immune activation. The Human Papillomavirus (HPV) vaccines is an example of an FDA-approved VLP vaccine. This vaccine has proven to be highly effective[2]. However, VLPs do have limitations. Their production requires expression and self-assembly of capsid proteins in host organisms, a process that can

be challenging to reproduce and scale up[46]. Additionally, VLPs can induce anti-carrier immunity, potentially limiting their application for multiple pathogens[47,48].

We aim to make vaccines against these three toxin-producing bacteria. Our first vaccine is designed to prevent *C. difficile* infections. For *B. anthracis* and *C. botulinum,* which cause multiple forms of disease, we strategically selected specific disease manifestations to target. Our *B. anthracis* vaccine aims to prevent inhalation anthrax due to its severity and potential bioterrorism risk[49]. For *C. botulinum*, we focused on preventing food-borne botulism for two key reasons. First, since our vaccine targets the botulism toxin rather than the spore, it has the highest probability of success against a toxin-mediated pathology[50]. Second, food-borne botulism represents one of the most common forms of human botulism, indicating a clear clinical need for preventative measures[50].

In this study, we developed an innovative nanoparticle vaccine platform by combining ultrasmall gold nanoparticles with iron-oxide nanoparticles, termed Vaccine Antigen Mimicry (VAM). The gold nanoparticles on the surface are strategically spaced approximately 5 to 10 nm apart, precisely implementing the “density code” principle that optimizes BCR clustering and activation. This spatial arrangement facilitates multivalent engagement of BCRs, enabling efficient crosslinking necessary for initiating B cell signaling cascades. The controlled antigen spacing is critical for stimulating B cell antigen presentation-mediated B/CD4 T cell crosstalk, both in vitro and in vivo[51,52]. The VAM platform’s ideal size, optimized antigen density, and enhanced BCR engagement collectively resulted in significantly higher antibody production compared to controls lacking these features. Notably, we demonstrated this enhanced antibody production across three distinct vaccines, each utilizing B/CD4 epitopes from different bacterial pathogens, underscoring the versatility of our platform. These results highlight the potential of our nanoparticle vaccine design in eliciting robust and broad-spectrum immune responses against various bacterial pathogens through the strategic implementation of virus-inspired structural principles.

2.3 Materials and Methods

2.3.1 Mice

All animal experiments were conducted according to the protocols approved by the University of Michigan Committee on Use and Care of Animals (UCUCA). C57BL/6J mice aged 6-8 weeks were purchased from Jackson Laboratories.

2.3.2 Preparation of Nanoparticles

A ligand exchanged procedure was used to modify the surface of oleic acid-functionalized iron oxide nanoparticles (Ocean Nanotech) **(Figure 2.1)**. 1mg of oleic acid nanoparticles were resuspended in ethanol. Equal molar amounts (.0068 mmol) of ligands were dissolved in dichloromethane and added to the ethanol/oleic acid nanoparticles. The reaction was vortexed and added to a bath sonicator and allowed to react for one hour. To remove excess ligands, the reaction mixture was centrifuged (10000 RCF, 10 minutes). The supernatant was removed and 1mL of ethanol was added. The pellet was resuspended, and this washing step was repeated for a total of 3 times. The pellet was resuspended in water and collected via magnet. This step was repeated for a total of 3 times to remove excess organic solvent. After final magnet collection, the nanoparticles were resuspended in water and stored in dark at 4°C.

To prepare gold nanodots, 500mM Na_2S solution (in water) was aged in the dark for 48hrs. The aged solution was diluted to 1mM. A 100mM solution of chloroauric acid ($HAuCl_4$) was diluted to 2mM. Na_2S (1mM) and $HAuCl_4$ (2mM) were added 3mL:1mL, respectively, and gently mixed. The mixture was incubated overnight at 4°C in the dark.

1mg of functionalized VAM was mixed with 3mL of gold nanodots and incubated overnight at 4°C. The mixture was added to a magnet and kept at 4°C overnight. The supernatant was removed, and the purified nanoparticles were resuspended in water and stored at 4°C.

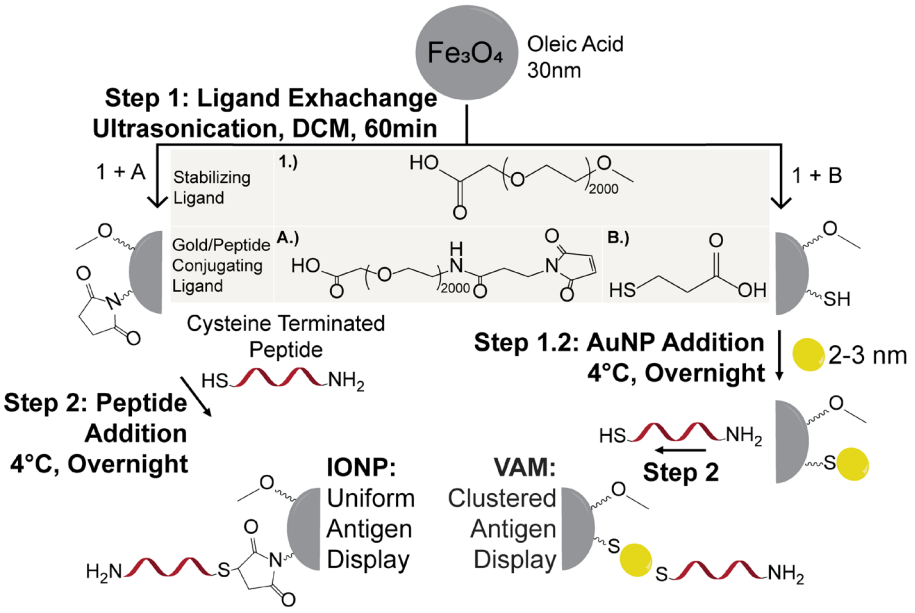


Figure 2.1 Ligand exchange procedure and nanoparticle formulation process

2.3.3 Peptide Selection and Conjugation

C. difficile produces two toxins TcdA and TcdB. Both toxins are composed of four different domains: Glucosyltransferase Domain (GTD), Pore Forming Region (PFR) Auto processing Domain (APD) and the Receptor Binding Domain (RBD). We chose one B cell and one CD4 epitope from TcdA and TcdB, for a total of 4 peptides. Our selection prioritized epitopes from the RBD, as antibodies targeting this region can prevent toxin binding to host cells, thus neutralizing toxin activity before cellular entry occurs[53]. Additionally, these epitopes have demonstrated strong immunogenicity and protective capacity in previous vaccination studies.

For *B. anthracis* which produces three different toxins: Protective Antigen (PA), lethal factor (LF), and the Edema Factor (EF). We strategically focused exclusively on the PA component. This decision was based on PA's critical role as the cellular binding component required for both LF and EF entry into host cells. By targeting PA alone, we could potentially neutralize the pathogenic effects of all three toxin components simultaneously[54]. We selected two B cell and two CD4 epitopes from PA regions that are highly conserved across strains and have been shown to elicit neutralizing antibodies in previous studies.

C. botulinum produces botulinum neurotoxin (BoNT) and it has seven different serotypes: BoNT/ A/ B/ C/ D/ E/ F/ G, with BoNT/A being responsible for the most

severe and prolonged human cases[55]. We selected three B cell and two CD4 epitopes from BoNT/A, focusing primarily on the receptor-binding domain and the catalytic domain. These specific regions were chosen because antibodies targeting the receptor-binding domain can prevent neuron entry, while those targeting the catalytic domain can neutralize the toxin's enzymatic activity even if it enters cells[56].

All peptides were ultimately chosen based on three key criteria: (1) demonstrated immunogenicity in previous studies, (2) conservation across bacterial strains to provide broad protection, and (3) accessibility to antibodies in the toxins' native conformation. These specific epitopes have been documented to elicit protective immune responses in various experimental models as detailed in (**Table 2.1**).

Cysteine terminally modified peptides in equal amounts were added to IONP or VAM (5:1 weight ratio, respectively) and incubated overnight at 4°C. The resulting nanoparticles were purified by centrifugation (10,000 RCF, 4°C, 30 minutes). Peptide conjugation was determined via modified fluorescamine peptide quantification assay. Quantification was performed using a standard curve with increasing peptide concentration. Peptides per nanoparticle was determined by converting peptide conjugation (g peptide/ g Fe) using the molar mass of the peptides and surface area of a sphere **(Appendix Table A.2).**

Table 2.1 Bacterial toxin peptides used in this study

Pathogen	Amino Acid Sequence	Antigen
C. difficile	CDDDGKKYYFNTNTSIAST[57]	TcdA RBD
	CDDDTGWQTIDGKKYYFNTNTAEAAT[58]	
	CDDDNQSGVLQTGVFSTEDGFKYFAPANTL[59]	TcdB RBD
	CDDDFTDEYIAATGSVIIDGEEYY[57]	
B. anthracis	CDDDKLPLYISNPNYKVNVYAVT[60]	PA
	CDDDAPIYNVLPTTSLVLGKNQTL[60]	
	CDDDGLLLNIDKDIRKILSGYIVEIEDTEGLKE[61]	
C. botulinum	CDDDHGNAEVHASFFDIGGS[62]	BoNT/A
	CDDDEVILKNAIVYNSMYENFST[63]	
	CDDDNNIMFKLDGCRDTHRYIWI[63]	
	CDDDSRTLGCSWEFIPVDDGWGERPL[63]	
	CDDDENFSTSFWIRIPKYFNSIS[63]	
	CDDDNKFLNQCSVSYLMNSMIPY[63]	

Abbreviations: TcdA, Toxin A; TcdB, Toxin B; RBD, Receptor Binding Domain; PA, Protective Antigen; BoNT/A, Botulinum Neurotoxin Type A

2.3.4 Characterization of Nanoparticles

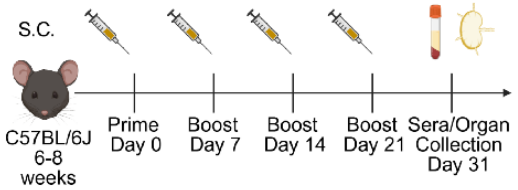
VAM and IONP were imaged by transmission electron microscopy using the Thermo Fisher Talos F200X G2 S/TEM. ImageJ software was used to quantify the actual particle size of AuNPs and VAM, estimate the distance between AuNPs on VAM, and to extrapolate the total AuNPs on the whole surface of VAM **(Appendix Table A.3)**. Briefly, by counting the number of AuNPs in one circle of known area, we can calculate the density of AuNPs/ nm^2. From here, we can estimate the total number of AuNPs per VAM by using the surface area of a sphere and known size of VAM. We can than determine the number of AuNPs/μ^2, by dividing the number of AuNPs per VAM by the surface area of VAM, and then converting nm^2 to μ^2. The hydrodynamic particle size, polydispersity index, and zeta-potential of all formulations in water at 25°C was evaluated with the Malvern Zetasizer nano-ZS using dynamic light scattering and phase analysis scatter, respectively.

2.3.5 BCR Binding

BCR binding was investigated according to our previous work[64]. Briefly, splenocytes were harvested from QM mice and B cells were isolated through negative selection using the EasySep Mouse B Cell Isolation Kit (STEMCELL: 19854). Isolated B Cells (5×10^6 cells/mL) were then incubated with 20μg/mL Alexa Fluor 488-AffiniPure Fab Fragment Goat Anti-Mouse IgM (μ Chain Specific) on ice for 30 min in the dark (Jackson: 115-167-020). Cells were washed and then incubated with antigen (equal amount of epitope, 20 nM, Cy3 labeled, and NP conjugated) for 5min at 37°C. Cells were then fixed and permeabilized, and then incubated with Alexa Fluor Plus 405 phalloidin on ice for 2 h. After two washes, the cells were plated onto eight-well glass chambers pretreated with 0.1% poly l-lysine (LabTech II) on ice for at least 4 h before confocal imaging.

2.3.6 Immunization and Sera Collection

At Day 0, mice were immunized with 50μg of peptide plus 10μg of c-di-GMP for all formulation types **(Figure 2.2)**. Mice were boosted three times at 7-day intervals with the same dosage of peptide and adjuvant (day 7,14, 21). To evaluate serum antibody titers, blood was collected via submandibular puncture at day 31. Serum was separated from whole blood by allowing it to clot for 30 minutes at room temperature and then centrifuging for 15 minutes at 2500 RCF.



Antigen(s)	Vaccine Group (n=4)	Adjuvant (c-di-GMP)
C. difficile TcdA/ TcdB	Soluble peptide only (50μg)	10μg
	AuNP (10μg) + 50μg peptide	10μg
	IONP (10μg) + 50μg peptide	10μg
	VAM (10μg) + 50μg peptide	10μg
B. anthracis (PA)	Soluble peptide only (50μg)	10μg
	IONP (10μg) + 50μg peptide	10μg
	VAM (10μg) + 50μg peptide	10μg
C. botulinum (BoNT-A)	Soluble peptide only (50μg)	10μg
	IONP (10μg) + 50μg peptide	10μg
	VAM (10μg) + 50μg peptide	10μg

Figure 2.2 Vaccine groups and formulations used for the in vivo antibody production study

2.3.7 Determination of Antibody Titers

The presence of serum IgG and of the subtypes IgG1 and IgG2b specific to vaccine peptide was determined by indirect ELISA. Nunc Immuno Maxisorp Plates were coated overnight at 4°C with peptides (100µL, 125µg/mL) in coating buffer (100mM carbonate buffer, pH 9.4). Plates were washed three times with 200µL of 1X PBS, pH 7.4, 0.05% Tween20 (PBST) and blocked with 200µL of 1% BSA in PBS for 60 min at 37°C. Contents were removed and sera samples were serially diluted in 0.1% BSA in PBS and then 100µL were added to each well and incubated for 120 min at 37°C. Plates were washed 4 times with PBST and then HRP-conjugated goat anti-mouse IgG (1:2500 diluted with 0.1% BSA in PBS) was added to each well and incubated for 1hr at 37°C. Plates were washed 4 times with PBST and incubated with 50µL of 3,3',5,5'-tetramethylbenzidine (1-Step Ultra TMB-ELISA Substrate Solution) until desired color developed. The reaction was stopped with 50µL of 0.1M sulfuric acid. Absorbance was measured at 450 nm on a plate reader. This method was repeated with IgG1 and IgG2b isotypes diluted according to the manufacturer recommendations. The titer was reports as the highest fold dilution that gave an absorbance value of 0.2. The negative control (no sera) had an absorbance value of 0.1, so we chose the cutoff to be two times higher than the control. Samples were tested in duplicate.

2.3.8 Statistical Analysis

Data are expressed as the mean ± standard deviation (SD), unless otherwise specified. Two groups were compared using the unpaired Student's t-test. Means of multiple groups were compared with one-way analysis of variance (ANOVA) followed by Tukey's post hoc pairwise comparison. All probability values are two-sided, and $p < 0.05$ was considered statistically significant. Statistical analyses were carried out using the GraphPad Prism 10 software package.

2.4 Results

2.4.1 Generation of Virus Antigen Mimicry Nanoparticles

A ligand exchange procedure was employed to modify the surface of oleic acid coated iron oxide nanoparticles (Ocean NanoTech, 30nm) **(Figure 2.1)**. One ligand, 3-mercaptopropionic acid, has a carboxylic acid group that binds to the iron oxide surface.

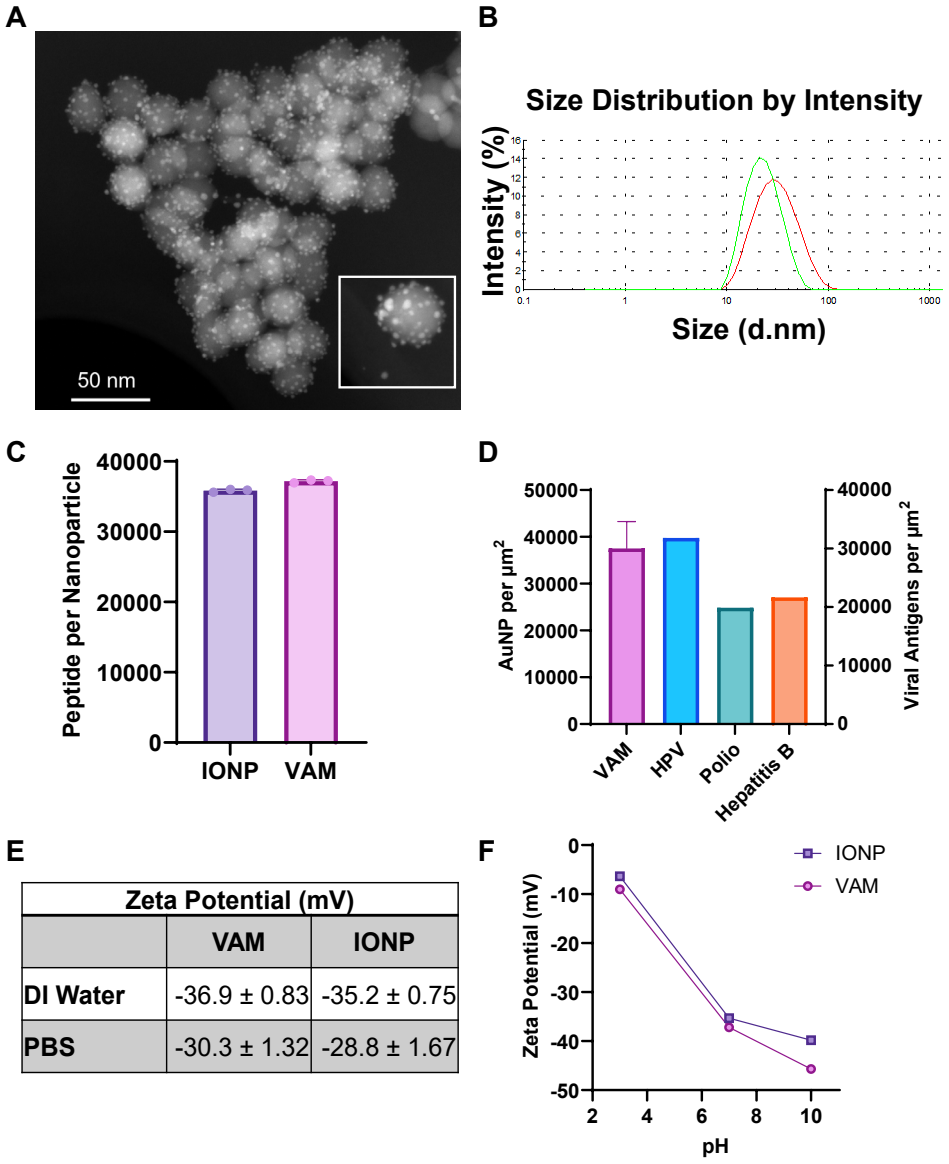
On the other end of the ligand is a thiol moiety, which allows us to conjugate ultra-small gold nanoparticles (AuNP, 2-3nm) to the iron oxide surface. This results in a hybrid Au@Fe nanoparticle platform, with antigen clusters, termed Virus Antigen Mimicry nanoparticle (VAM). To afford aqueous stability, a second ligand was included, COOH-PEG(2K)- Methoxy. A second control nanoparticle was produced, which lacks the antigen cluster feature, but has a similar surface chemistry (IONP). It has a COOH-PEG(2K)- Maleimide ligand instead of 3-mercaptopropionic acid. Separately, AuNPs were prepared via reduction of chloroauric acid in aged sodium sulfide[65]. The resulting AuNP solution was added to VAM and incubated overnight at 4°C.

2.4.2 Characterization and Evaluation of Viral Mimicry Features

The addition of AuNPs to the VAM core was confirmed via transmission electron microscopy (TEM) (**Figure 2.3A**). From the TEM images the VAM core and AuNP diameters were quantified to be 27.1 ± 0.1 nm and 2.2 ± 0.5 nm, respectively. The total number of AuNPs on the entire surface of one VAM particle was estimated to be 91 ± 8, which we can extrapolate from the TEM image. With the number of AuNPs per VAM known, we can calculate the AuNPs per unit area, which ranged from about 32,000 to 43,000 AuNPs per square micron (**Figure 2.3D**). This number is comparable to the antigen density reported for viruses (e.g. Human Papillomavirus, ~ 30,000 antigens per μ^2)[34]. Lastly, TEM visualization revealed the spacing between AuNPs was 5.6 ± 1.8 nm, which aligns with the ideal 5-10 nm distance for B-cell receptor binding[66].

Surface charge and size are also important factors for vaccine development[67]. VAM and IONP were analyzed by DLS prior to peptide loading to ensure similar physicochemical properties. VAM and IONP had hydrodynamic diameters of 24.11 ± 9.28 and 33.2 ± 15.68, respectively. Polydispersity index (PDI) was 0.165 and 0.176, respectively (**Figure 2.3B**). Zeta potential was determined in various pH and buffers. The zeta potential for VAM was shown to be -36.9 ± 0.83 mV (DI water), -30.3 ± 1.32 mV (PBS) (**Figure 2.3E**), -9 ± 1 mV (pH 3), -37 ± 1 mV (pH 7), and -45 ± 3 mV (pH 10) (**Figure 2.3F**). Whereas for IONP the zeta potential was -35.2 ± 0.75 mV (DI water), -28.8 ± 1.67 mV (PBS), -6 ± 0.4 mV (pH 3), -36 ± 0.3 mV (pH 7), and -40 ± 1 mV (pH 10).

Optimal antigen spacing and organization is crucial for effective viral mimicry, so peptide loading was evaluated for the two groups[34]. We chose *C. difficile* TcdA and TcdB peptides, which contain B cell and CD4 epitopes. These peptides were chosen due to their proven antigenicity in previously published works. A cysteine containing terminal flank (CDD) was added to all peptide antigens to facilitate facile conjugation to VAM via the Au-S linkage. Peptide loading was determined to be 37,179 ± 202 peptides per VAM particle and 34,130 ± 491 peptides per IONP particle (**Figure 2.3C**). Overall, VAM and IONP have similar size, surface charge, and peptide amounts.



Zeta Potential (mV)		
	VAM	IONP
DI Water	-36.9 ± 0.83	-35.2 ± 0.75
PBS	-30.3 ± 1.32	-28.8 ± 1.67

Figure 2.3 Characterization of VAM and IONP and viral mimicry. (A) Transmission electron microscopy (TEM) images of VAM. Scale bar = 50 nm. (A-insert) High resolution TEM of single nanoparticle. (B) Intensity based size distributions of VAM (green) and IONP (red) by Dynamic Light Scattering (DLS). (C)

Quantification of peptide loading on VAM and IONP determined by a modified fluorescamine fluorescent detection assay; data represent mean ± SD, n = 3. (D) Mathematical modeling of theoretical density (nanoparticles per unit area) of AuNPs on VAM surfaces and as compared to the known antigen density on viral capsids, data represent mean ± SD, n = 3. (E) Zeta potential of VAM and IONP in DI water and PBS at 25°C, data represent mean ± SD, n = 3. (F) Zeta potential of VAM and IONP at different pH at 25°C, data represent mean ± SD, n = 3. SD, standard deviation; DI, deionized water; PBS, phosphate buffered saline.

2.4.3 VAM demonstrates superior BCR Binding

To evaluate how antigen spatial organization influences BCR engagement, we compared the binding efficiency of VAM versus IONP. Both VAM and IONP were labeled with Cy3 and hapten, where hapten was conjugated to the end of B cell epitopes. The nanoparticles were then incubated with hapten-specific B cells from the spleen of QM transgenic mice (**Figure 2.4A**). The results showed that hapten labeled-VAM (red) efficiently engaged with BCRs (stained with FITC-labeled antibody, green) (**Figure 2.4B**). Interestingly, IONP, despite having similar antigen density and size, demonstrated significantly lower BCR engagement. The enhanced BCR interaction with VAM is evidenced by the increased colocalization (yellow) present in the VAM images. This data suggests that the specific spatial arrangement of antigens in clusters, rather than merely their presence or total quantity, is critical for effective BCR engagement and subsequent B cell activation.

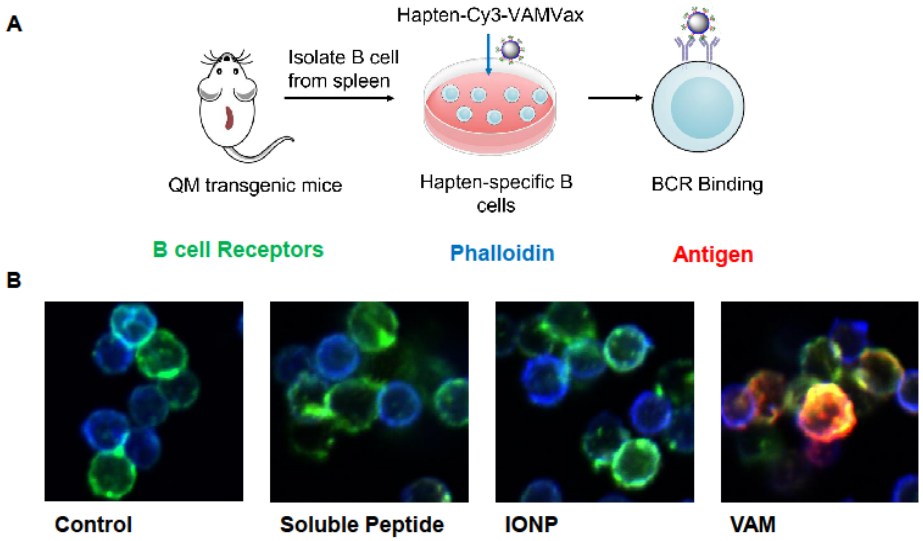


Figure 2.4 Enhanced BCR Engagement of VAM. (A) Diagram of experimental design. (B) Confocal image of Cy3 and hapten labeled VAM (red) binding (yellow) with BCR (green) in hapten-specific B cells from QM mice splenocytes, compared to control (no antigen), soluble peptide (B/CD4 antigen labeled with Cy3 and hapten, IONP (Cy3 and hapten labeled) with similar antigen amount (all formulations are 20nM

antigen). Blue, phalloidin stain of actin filaments; green, B cell receptor staining using Alexa Fluor 488-AffniPure Fab Fragment Goat Anti-Mouse IgM (μ Chain Specific) antibody; red: Cy3 labeled CD4/B-hapten epitope. BCR, B cell receptor.

2.4.4 VAM Demonstrates Superior Antigen-Specific Antibody Production in Mice

The most important part of viral mimicking nanoparticles is its function of activating B-cells for antigen specific antibody production[68]. We wanted to test if the antigen clusters from VAM impact the in-vivo antibody production compared to IONP, which has very similar physicochemical properties to VAM, but has a uniform antigen display. To evaluate the antibody production for the *C. difficile* vaccine, C57BL/6 were immunized with VAM, IONP, AuNP or soluble peptide (50μg peptide, 10μg c-di-GMP) at day 0, and boosted three times at 7-day intervals. Serum was analyzed 10 days after the last dose for antigen-specific IgG and the antigen-specific IgG isotypes: IgG1 and IgG2b. Serum analysis revealed that for the *C. difficile* vaccine, VAM had an antigen-specific IgG antibody production 2-4-fold higher than IONP **(Figure 2.5A)**. Specifically, VAM generated a 3-fold higher antigen-specific IgG titer (42529 ± 5374 vs 18429 ± 5493; $p < 0.0005$) a 2-fold higher antigen-specific IgG1 titer (10243 ± 2551 vs 4933 ± 1314; $p < 0.005$) and a 4-fold higher antigen-specific IgG2b titer (34862 ± 509 vs 9499 ± 1582; $p < 0.0005$) as compared to IONP. Similarly, VAM generated a 5-fold higher antigen-specific IgG titer (42529 ± 5374 vs 8780 ± 1672; $p < 0.0005$) an 8-fold higher antigen-specific IgG1 titer (10243 ± 2551 vs 1235 ± 236; $p < 0.0005$) and a 9-fold higher antigen-specific IgG2b titer (34862 ± 509 vs 3821 ± 1047; $p < 0.0005$) as compared to AuNP. Lastly, VAM generated a 14-fold higher antigen-specific IgG titer (42529 ± 5374 vs 2880 ± 818; $p < 0.0005$) a 10-fold higher antigen-specific IgG1 titer (10243 ± 2551 vs 1057 ± 56; $p < 0.0005$) and a 13-fold higher antigen-specific IgG2b titer (34862 ± 509 vs 2680 ± 346; $p < 0.0005$) compared to soluble peptide. These data suggest that virus-like features are favorable for antibody production.

After establishing that viral-like features could enhance antibody production, we wanted to test the versatility of our VAM platform and see if we could replicate the results with antigens for other bacterial pathogens. Peptide antigens for *B. anthracis* and *C. botulinum* were chosen in the same manner as *C. difficile* and contain the same cysteine modification to facilitate binding to VAM. The same study was repeated as

above, only utilizing antigens from *B. anthracis* protective antigen (**Figure 2.5B**). VAM had an antigen-specific IgG antibody production 2-3-fold higher than IONP. Specifically, VAM generated a 3-fold higher antigen-specific IgG titer (28009 ± 5484 vs 10816 ± 958; $p < 0.0005$) a 4-fold higher antigen-specific IgG1 titer (10463 ± 578 vs 2927 ± 825; $p < 0.005$) and a 2-fold higher antigen-specific IgG2b titer (17254 ± 636 vs 7821 ± 385; $p < 0.0005$) as compared to IONP. Next, VAM generated a 14-fold higher antigen-specific IgG titer (28009 ± 5484 vs 1984 ± 197; $p < 0.0005$) a 19-fold higher antigen-specific IgG1 titer (10463 ± 578 vs 558 ± 157; $p < 0.0005$) and a 13-fold higher antigen-specific IgG2b titer (17254 ± 636 vs 1344 ± 206; $p < 0.0005$) compared to soluble peptide. Continuing, we also repeated the study for a third time, utilizing antigens from *C. botulinum* neurotoxin type A (**Figure 2.5C**). VAM had an antigen-specific IgG antibody production 4-fold higher than IONP. Specifically, VAM generated a 4-fold higher antigen-specific IgG titer (10112 ± 454 vs 811 ± 177; $p < 0.0005$) a 4-fold higher antigen-specific IgG1 titer (2733 ± 588 vs 628 ± 160; $p < 0.005$) and a 4-fold higher antigen-specific IgG2b titer (7102 ± 806 vs 1744 ± 368; $p < 0.0005$) as compared to IONP. Next, VAM generated a 12-fold higher antigen-specific IgG titer (10112 ± 454 vs 811 ± 177; $p < 0.0005$) a 14-fold higher antigen-specific IgG1 titer (2733 ± 588 vs 200 ± 26; $p < 0.0005$) and a 10-fold higher antigen-specific IgG2b titer (7102 ± 806 vs 603 ± 168; $p < 0.0005$) compared to soluble peptide.

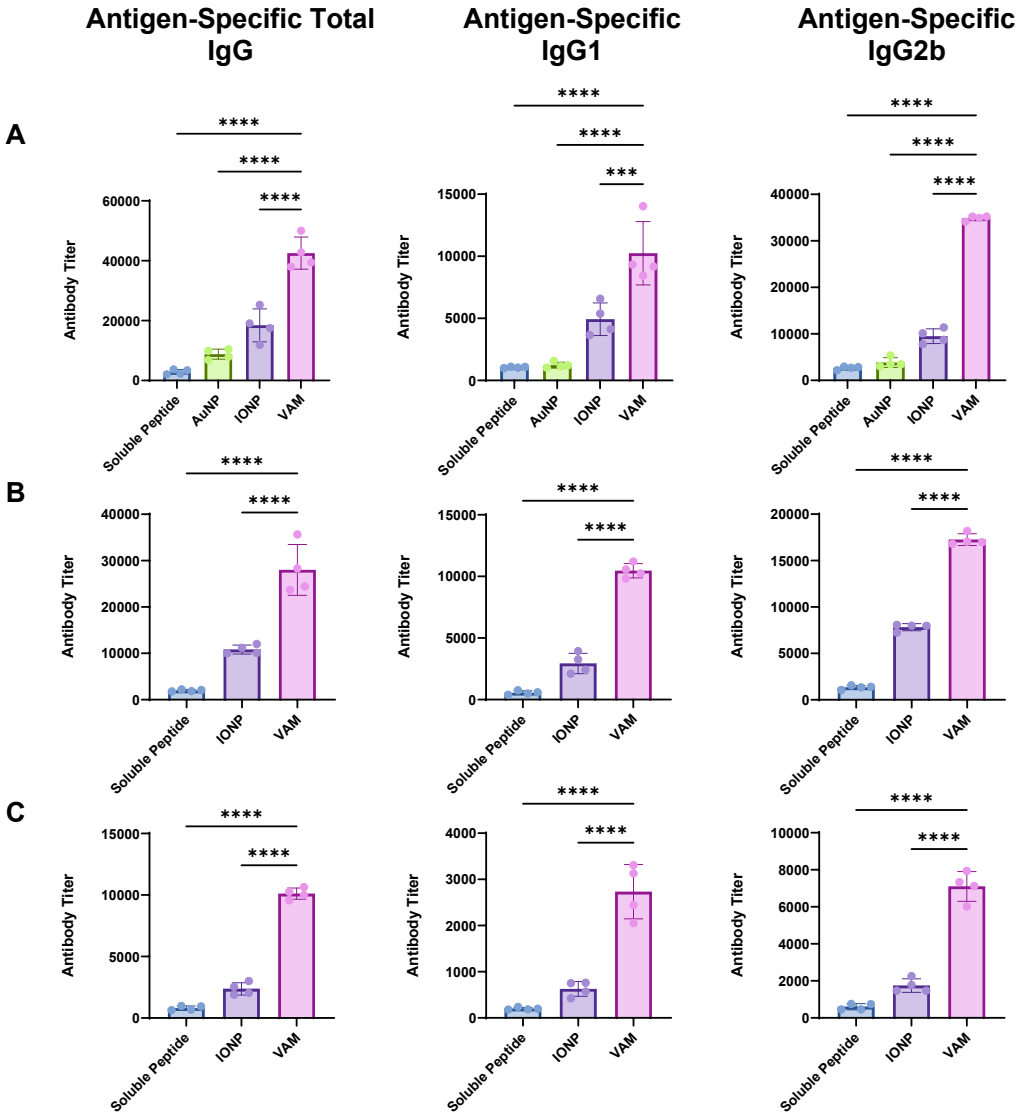


Figure 2.5 VAM demonstrates superior antigen-specific antibody production in mice. (A) Day 31 serum antigen-specific antibody titers (IgG, IgG1 and IgG2b) after C. difficile vaccine administration; data represent mean ± SD, n = 4. (B) Day 31 serum antigen-specific antibody titers (IgG, IgG1 and IgG2b) after B. anthracis vaccine administration data represent mean ± SD, n = 4. (C) Day 31 serum antigen-specific antibody titers (IgG, IgG1 and IgG2b) after C. botulinum vaccine administration data represent mean ± SD, n = 4. Statistical comparisons are based on one-way ANOVA, followed by post hoc Tukey's pairwise comparisons. The asterisks denote statistical significance at the level of *** $p < 0.001$. **** $p < 0.0001$. ANOVA, analysis of variance; SD, standard deviation.

2.5 Discussion

Vaccines play a crucial role in preventing infectious disease. However, the limited efficacy of certain vaccine types necessitates the development of alternative platforms. In our study, we successfully developed a versatile Virus Antigen Mimicry nanoparticle

(VAM) platform. VAM features gold nanoparticles on its surface with an antigen density comparable to natural virus spikes (>10,000 antigens/micron)[66]. Moreover, VAM's optimal size (<200 nm) combined with its structural organization enhanced B-cell receptor (BCR) engagement and antibody production. This innovative platform not only demonstrates improved immunogenicity but also offers the advantage of potential large-scale manufacturing due to the readily available starting materials.

The VAM platform's key feature is its peptide versatility, as demonstrated in our study where three different sets of peptides were successfully conjugated to VAM to create three distinct vaccines, all producing high antibody titers. This versatility contrasts with traditional vaccine approaches, which often face limitations. As mentioned previously, common bacterial vaccines typically utilize toxoids. While toxoid vaccines are immunogenic, toxins exhibit strain variability, potentially leading to antibodies that fail to recognize and neutralize different toxin variants[69].

Traditional protein Virus-Like Particles (VLPs), such as those used in the human papillomavirus vaccine, have their own constraints, as not every antigen can self-assembled into a VLP[70]. Moreover, these vaccine types often require new formulations or extensive engineering to incorporate new peptides, a process that can be both costly and time-consuming[45]. In contrast, VAM offers a more flexible approach, requiring no formulation or engineering changes for new or different peptides. The only requirement is that the peptides must be cysteine-terminated for conjugation to the VAM surface.

It’s important to note a limitation in our immunization study: we only used peptides from toxin-based epitopes. We initially selected this approach for several strategic reasons. First, bacterial toxins often represent the primary virulence factors directly responsible for disease pathology, making them logical targets for neutralizing antibodies[71,72]. Second, toxin epitopes typically have conserved structures essential for their function, reducing the risk of antigenic variation[69,73]. Third, this approach allowed us to evaluate VAM’s capabilities using well-characterized antigens with established relevance to pathogenesis. Despite these advantages, toxin-based vaccines do not reduce bacterial colonization and have limited efficacy against the full spectrum of disease mechanisms. However, VAM’s versatility allows for the use of virtually any peptide, opening possibilities for developing vaccines against bacteria with various

antigens. This flexibility makes VAM highly customizable, potentially useful for creating vaccines against multiple types of pathogens or even multivalent vaccines targeting different strains or species simultaneously. Such adaptability could significantly streamline the vaccine development process, offering a more efficient and cost-effective approach to addressing diverse immunological challenges.[74,75].

In our antibody production study, we compare VAM to three alternatives: free peptide antigen, AuNP, and IONP. These comparisons help us evaluate the effectiveness of VAM in terms of antigen presentation, size optimization, and uniform antigen display. While free peptide antigens can generate antibodies, they are typically less immunogenic than when conjugated to a nanocarrier. This reduced immunogenicity stems primarily from their tendency to induce inhibitory signaling and immune tolerance rather than activation when presented without appropriate co-stimulatory signals or inflammatory context[76,77]. Additionally, free peptides face challenges with rapid degradation, limited lymph node delivery, and suboptimal presentation to immune cells[78]. In contrast, nanoparticle carriers can enhance immunogenicity by providing a multivalent display of antigens alongside danger signals that promote productive immune activation rather than tolerance[35,79].

The size of nanoparticles plays a critical role in their effectiveness as vaccine carriers, with the optimal range for lymph node delivery and retention generally considered to be 10-100 nm[80]. This is due to the physiology of lymphatic vessels, where particles enter via endothelial cell junctions (~100 nm)[81]. Particles smaller than 10 nm can enter both blood and lymphatic vessels but typically exhibit poor lymph node retention due to their ability to rapidly diffuse across vessel walls and undergo renal clearance[82]. Conversely, particles larger than 200 nm have poor lymphatic uptake and primarily remain at the injection site, where they may be processed by local immune cells rather than reaching secondary lymphoid organs[83].

While IONP and VAM have similar size, surface charge, and peptide-per-nanoparticle ratios, they differ in antigen display architecture. VAM features gold nanoparticles on its surface with antigen clusters spaced at 5-10 nm, while IONP has peptides conjugated directly to the iron oxide core. The enhanced BCR engagement and subsequent antibody production observed with VAM could be attributed to several

potential factors, including the specific spatial organization of antigens, properties of the gold surface that may affect peptide orientation or conformation, or differences in how these nanoparticles interact with immune cells. Our experimental design focused on demonstrating VAM's efficacy rather than isolating the precise mechanism of its superior performance.

In conclusion, VAM's superior performance in antibody production (3-14x higher than other groups) can be attributed to its optimal size for lymph node delivery (~30 nm) and enhanced BCR engagement, though further studies would be needed to definitively identify which specific architectural features are most critical to its immunogenicity. These features collectively contribute to VAM's improved performance compared to free peptides, AuNPs, and IONPs, making it a promising candidate for vaccine development.

2.6 Conclusions

In our study, we successfully developed a virus antigen mimicry nanoparticle (VAM) utilizing readily available materials. VAM was engineered with specific structural features inspired by viruses, including optimal size for lymphatic trafficking and strategic antigen spacing of 5-10nm. This spatial organization of antigens appears to enhance BCR engagement through multivalent interactions and receptor clustering, leading to more efficient B cell activation compared to controls. VAM demonstrated enhanced immune cell activation and class-switched antibody production across three different bacterial toxin targets, highlighting the platform's versatility. While our results indicate that the specific architectural arrangement of antigens contributes to VAM's performance, future studies should further elucidate the precise mechanisms underlying this enhanced immunogenicity.

Our study did not investigate protective efficacy, nor the functional activity of the antibodies produced from vaccination. Future studies should aim to determine if antibodies produced from vaccination can neutralize toxin activity or reduce bacterial burden in challenge models. Additionally, exploring how VAM's antigen presentation influences different aspects of the immune response beyond antibody production could provide valuable insights for vaccine design. Overall, this work provides a promising and adaptable nanoparticle vaccine platform that implements key structural principles for enhanced immunogenicity against bacterial infections.

2.7 Supporting Information

The supporting information and tables from Chapter 2 are in Appendix A

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Chapter 3 Exploring Oral Vaccine Delivery: Development and Evaluation of Enteric-Coated Capsules for Mucosal Immunity Stimulation

3.1 Abstract

This study investigated enteric-coated capsules for oral vaccine delivery using virus antigen mimicry (VAM) nanoparticles to stimulate mucosal immunity. Mucosal surfaces, the primary entry point for most pathogens, benefit from IgA antibodies that parenteral vaccines rarely induce. We optimized enteric-coated gelatin capsules to protect lyophilized VAM from gastric degradation while enabling intestinal release. Dissolution assays determined that four polymer coatings provided adequate gastric protection with rapid dissolution at intestinal pH. Cryoprotectants (15% HSA and 2% sucrose) successfully prevented lyophilization-induced nanoparticle aggregation. Despite these formulation successes, neither oral gavage of aqueous VAM nor capsule delivery of lyophilized VAM elicited detectable antigen-specific antibody responses in mice and hamsters, respectively. Control groups receiving subcutaneous injections showed expected immune responses, confirming VAM immunogenicity when administered parenterally. This work establishes foundational approaches for oral vaccine delivery while highlighting challenges in stimulating mucosal immunity. Future studies should focus on optimizing capsule dissolution kinetics, increasing antigen dosing, and investigating nanoparticle stability in gastrointestinal conditions.

3.2 Introduction

Mucosal surfaces are the main point of entry for 90% of human pathogens[1]. Mucosal surfaces represent the first line of protection against infections. The mucosa traps pathogens and foreign substances, preventing them from reaching further into tissues. Indirectly, this can reduce shedding of pathogen in mucosal surfaces, thereby reducing secondary transmission of pathogens[2]. A vaccine that induces a strong

mucosal immune response could enhance this natural protection, further reducing the number of infections[3,4].

Most vaccines are delivered parentally and often induce IgG antibodies[5,6]. IgG is primarily found in blood and extracellular fluid, mainly operating in tissues. IgG can efficiently opsonize pathogens prior to phagocytosis and strongly activates the complement system[7,8]. IgG is typically ineffective at mucosal sites and parental vaccines are typically poor inducers of mucosal immunity, limiting protection at mucosal surfaces[9,10]. Not to mention administration of parental vaccines requires needles, syringes, sharps disposal, trained staff to administer the vaccines. These requirements are costly and make mass vaccination programs harder for low-income areas, who are most vulnerable to infectious disease[11].

Vaccination via mucosal routes have been shown to elicit mucosal immunity the best[12,13]. One of the most widely studied routes for mucosal vaccines is oral administration. Oral vaccines alleviate many of the problems of parental vaccines[14,15]. Oral vaccines are often easier to administer, since self-administration is possible. Patient compliance is improved, since many people adverse to injections are more likely to take an oral vaccine. Most importantly, mucosal vaccines have the ability to produce both a systemic and mucosal immune response. A strong mucosal immune response provides protection at mucosal surfaces which can prevent pathogens from invading past mucosal tissues[16,17]. This study will focus on oral vaccine delivery to stimulate mucosal immunity.

A mucosal immune response can be broadly characterized as the production of IgA antibodies. IgA is the principal antibody in secretions, most importantly the epithelium of the gastrointestinal and respiratory tracts[2]. Oral vaccines will primarily induce immunity at mucosal tissues in the GI tract, further highlighting the relevance of this antibody. Unlike IgG, IgA poorly opsonizes and only weakly activates the complement system, so it doesn't induce inflammation[18]. IgA secreting plasma cells are found chiefly in the lamina propria and then the IgA antibodies can be transported to the lumen of the gut. IgA is secreted as a dimer or polymer, which is a complex of a secretory component (SC) that is cleaved from the extracellular portion of the polymeric Ig receptor[19,20]. Secretory IgA (sIgA) functions by directly cross-linking to pathogens,

preventing their adherence to host tissues and facilitating their elimination[21]. sIgA can also neutralize microbial toxins or enzymes at mucosal surfaces[22,23].

In order to produce IgA antibodies, an oral vaccine needs deliver intact antigen to the small intestine. The inductive site of the gut is the small intestine, particularly the Peyer's patches that contain M cells[24,25]. M cells continuously sample food and foreign antigens and transport them across the epithelium to antigen presenting cells (APCs)[26,27]. The dendritic cells (DCs) uptake, process and present to activate naïve $CD4^+$ T cells. These $CD4^+$ T cells activate localized B cells and class switch to become IgA^+ B cells[28,29].

While oral vaccines have many benefits, there are challenges that limit the success of many vaccine candidates. The biology of the GI tract alone poses many challenges. The pH is very acidic in the stomach and changes drastically along the entirety of the GI tract[30,31]. There are also proteolytic enzymes present[32]. These characteristics can degrade antigenic peptides and proteins. This is also one of the reasons oral vaccines typically need a higher dose of antigen. Larger doses might also trigger immune tolerance instead of a protective response[3]. Lastly, the GI tract constantly samples antigen, a vaccine needs to have the appropriate danger signals to stimulate the immune system. This typically requires the use of potent adjuvants as part of the oral vaccine formulation[33].

Despite the challenges of oral vaccines, there have been multiple approved by the FDA. Current U.S licensed vaccines include those for the prevention of cholera, rotavirus, and typhoid fever. Most of these vaccines utilize live attenuated pathogens to induce an immune response. These vaccines have been shown to produce sIgA and serum IgG, and provide protection against these diseases, demonstrating that oral vaccines can be an effective vaccine strategy[11].

In this study, we wanted to test if we could stimulate mucosal immunity with our virus antigen mimicry (VAM) nanoparticle platform, specifically in the form of antigen-specific IgA antibodies. We first tried dosing animals via oral gavage with our aqueous VAM solution, this did not induce any detectable antibody response (IgG or IgA antibodies). We then formulated a polymer solution to coat gelatin capsules, which resist degradation in the low pH of the stomach but dissolve rapidly in the higher pH of

the intestine. The use of capsules necessitates the lyophilization of VAM. By utilizing cryoprotectants, we were able to prevent lyophilization induced aggregation of VAM. Despite the successes of the dissolution assay and the cryoprotection study, the VAM capsule vaccine still failed to produce any antigen specific antibodies.

3.3 Materials and Methods

3.3.1 Mice

All animal experiments were conducted according to the protocols approved by the University of Michigan Committee on Use and Care of Animals (UCUCA). C57BL/6J mice aged 6-8 weeks were purchased from Jackson Laboratories.

3.3.2 Preparation of Nanoparticles

1mg of oleic acid-functionalized iron oxide nanoparticles (Ocean NanoTech) were resuspended in ethanol. Equal molar amounts (.0068 mmol) of ligands were dissolved in dichloromethane and added to the ethanol/oleic acid nanoparticles. The reaction was vortexed and added to a bath sonicator and allowed to react for one hour. To remove excess ligands, the reaction mixture was centrifuged (10000 RCF, 10 minutes). The supernatant was removed and 1mL of ethanol was added. The pellet was resuspended, and this washing step was repeated for a total of 3 times. The pellet was resuspended in water and collected via magnet. This step was repeated for a total of 3 times to remove excess organic solvent. After the final magnet collection, the nanoparticles were resus-pended in water and stored in dark at 4°C.

To prepare gold nanodots, 500mM Na_2S solution (in water) was aged in the dark for 48hrs[34]. The aged solution was diluted to 1mM. A 100mM solution of chloroauric acid ($HAuCl_4$) was diluted to 2mM. Na_2S (1mM) and $HAuCl_4$ (2mM) were added 3mL:1mL, respectively, and gently mixed. The mixture was incubated overnight at 4°C in the dark.

1mg of functionalized VAM was mixed with 3mL of gold nanodots and incubated overnight at 4°C. The mixture was added to a magnet and kept at 4°C overnight. The supernatant was removed, and the purified nanoparticles were resuspended in water and stored at 4°C.

Cysteine terminally modified HER2 peptides (CDDD-PESFDGDPASNTAPLQPEQLQ) were chosen as our model antigen. We chose this peptide because we have tested it in our previous studies and showed success using a similar viral mimicry nanoparticle platform[35,36]. HER2 peptides were added to IONP or VAM (5:1 weight ratio, respectively) and incubated overnight at 4°C. The resulting nanoparticles were purified by centrifugation (10,000 RCF, 4°C, 30 minutes). Peptide conjugation percentage was determined via modified fluorescamine peptide quantification assay. Quantification was performed using a standard curve with increasing peptide concentration.

3.3.3 Subcutaneous and Oral Gavage Immunization and Sera Collection in Mice

The nanoparticles were prepared as above, conjugated to peptide, mixed with adjuvant prior to administering the aqueous vaccine. The aqueous solution was administered to mice via oral gavage. At Day 0, mice were immunized with 50μg of peptide plus 10μg of c-di-GMP for all formulation types. Mice were boosted three times at 7-day intervals with the same dosage of peptide and adjuvant (day 7,14, 21). As a control, equal groups of mice were also immunized with the same vaccine via subcutaneous injection. To evaluate serum antibody titers, blood was collected via submandibular puncture at day 31. Serum was separated from whole blood by allowing it to clot for 30 minutes at room temperature and then centrifuging for 15 minutes at 2500 RCF. Feces were collected at endpoints. Feces were washed with PBS three times, after final wash they were diluted 1:9 with PBS, and centrifuged again (10,000 RCF, 10min). The supernatant was removed into a clean tube and used for antibody determination.

3.3.4 Dissolution Assay to Optimize Capsule Coating

2mg of powdered methylene blue was added to gelatin capsules, size M (Torpac). The capsules were dipped in a coating that consists of 12.5% EUDRAGIT L100 in isopropanol with triethyl acetate (TEC) (10% w/v) and 3% H2O[37]. The capsules were added to simulated gastric fluid without enzyme (0.2% NaCl in DI water, pH was adjusted to 1.2 with 0.1M HCl) and monitored visually for 5hrs. The capsule was then transferred to simulated intestinal fluid without enzyme (50mM monobasic potassium

phosphate in DI water, pH was adjusted to 6.8 with 0.1M NaOH) and observed visually for 2 hours. The dissolution of 1 coat up to 4 coats (allowing to dry completely between coats) were compared.

3.3.5 Cryoprotection Evaluation of VAM

VAM was freshly prepared and conjugated to peptides as described above. sucrose (2%) and Human Serum Albumin (HSA) (15%) were added to VAM vaccine formulations as cryoprotectants. The hydrodynamic diameter of VAM was determined by dynamic light scattering (DLS) before and after lyophilization.

3.3.6 Capsule Administration and Sera Collection in Hamsters

Due to the size of the capsule dosing applicator, hamsters were used to evaluate the immune response produced from administration of coated capsule containing the lyophilized vaccine. A dosing applicator provided by the manufacturer was used to dose the hamster, similar to oral gavage. At Day 0, hamsters were immunized with 250 μg of peptide plus 20 μg of c-di-GMP for all formulation types. Hamsters were boosted three times at 7-day intervals with the same dosage of peptide and adjuvant (day 7,14, 21). As a control, equal groups of hamsters were also immunized with the same vaccine via subcutaneous injection. To evaluate serum antibody titers, blood was collected via jugular vein at day 31. Serum was separated from whole blood by allowing it to clot for 30 minutes at room temperature and then centrifuging for 15 minutes at 2500 RCF. Feces were collected at endpoints. Feces were washed with PBS three times, after final wash they were diluted 1:9 with PBS, and centrifuged again (10,000 RCF, 10min). The supernatant was removed into a clean tube and used for antibody determination.

3.3.7 ELISA

The presence of serum IgG and fecal IgA was determined by indirect ELISA. Nunc Immuno Maxisorp plates were coated overnight at 4°C with peptides (100μL, 125μg/mL) in coating buffer (100mM carbonate buffer, pH 9.4). Plates were washed three times with 200μL of 1X PBS, pH 7.4, 0.05% Tween20 (PBST) and blocked with 200μL of 1% BSA in PBS for 60 min at 37°C. Contents were removed and sera samples were serially diluted in 0.1% BSA in PBS and then 100μL were added to each well and incubated for 120 min at 37°C. Plates were washed 4 times with PBST and then HRP-

conjugated goat anti-mouse IgG (1:2500 diluted with 0.1% BSA in PBS) was added to each well and incubated for 1hr at 37°C. Plates were washed 4 times with PBST and incubated with 50µL of 3,3',5,5'-tetramethylbenzidine (1-Step Ultra TMB-ELISA Substrate Solution) until desired color developed. The reaction was stopped with 50µL of 0.1M sulfuric acid. Absorbance was measured at 450 nm on a plate reader. This method was repeated with IgA diluted according to the manufacturer's recommendations. Pre immune sera were used to determine baseline cutoff values, values higher than cut off were determined positive and lower values were negative for antibody production. Samples were tested in triplicate.

3.3.8 Statistics

Data are presented as mean ± standard deviation (SD) unless otherwise specified. For comparisons between two groups, the unpaired Student's T-test was employed. When comparing multiple groups, one-way analysis of variance (ANOVA) was conducted, followed by post hoc Tukey's pairwise comparisons. All probability values are two-sided, with $p < 0.05$ considered statistically significant. Statistical analyses were performed using GraphPad Prism 10 software.

3.4 Results

3.4.1 Vaccines Administered Via Oral Gavage Were Unable to Evoke an Immune Response in Mice

We ultimately wanted to determine if a capsule was a necessary step in designing an oral vaccine, or if the nanoparticles alone could provide some protection against peptide degradation. To test if the vaccine (nanoparticle, antigen, and adjuvant) solution could produce a mucosal immune response, we immunized C57BL/6b mice via oral gavage with VAM, IONP, or soluble peptide (50 µg of HER2 peptide, 10 µg c-di-GMP, at days 0, and boosted three times at 7-day intervals (**Figure 3.1A**). Serum and feces were collected 10 days after the last dose and evaluated for antibody production.

As a control, we also performed subcutaneous injections of all the vaccine groups, to provide a baseline for antibody production. Unfortunately, all groups of mice receiving vaccines via oral gavage were negative for antigen-specific IgG antibodies in sera (**Figure 3.1B**). None of the groups had absorbance values higher than the cut off

value, which was determined as two times higher than the negative control. The subcutaneous vaccines demonstrated similar serum IgG trends, as shown previously. The antigen-specific IgG OD450 for VAM was 2-5-fold higher than controls (**Figure 3.1B**). Specifically, the IgG OD450 for VAM was 2-fold higher (1.35 ± 0.28 vs 0.64 ± 0.05), 5-fold higher (1.35 ± 0.28 vs 0.27 ± .03) than IONP and soluble peptide groups, respectively. Neither SC nor oral gavage vaccine groups were positive for antigen-specific IgA antibodies **(Figure 3.1C).**

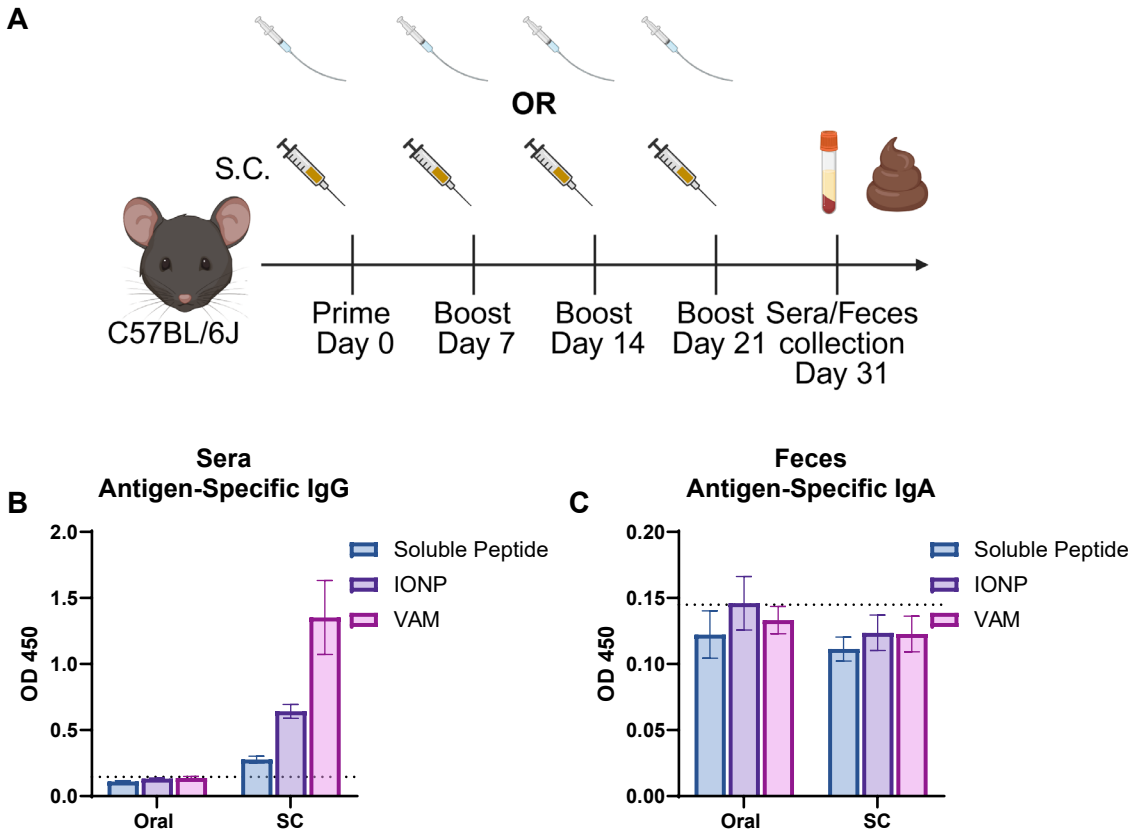


Figure 3.1 Comparing antibody production of vaccines utilizing different delivery strategies (subcutaneous injection vs oral gavage). (A) Animal study immunization and sampling timeline. Antigen- specific antibody OD 450 values after administration of HER2 vaccine; data represent mean ± SD, n = 4. (B) IgG in sera (C) IgA in Feces. Dotted lines represent cut-off values, which was calculated from twice the value of the negative control. SC, subcutaneous injection.

3.4.2 Four Dip Coatings of Gelatin Capsules Were Necessary to Resist Dissolution in Low pH and Dissolve Quickly in Higher pH

An oral vaccine needs to be able to travel to the small intestine, where antigen uptake occurs. The vaccine should be protected from the low pH of the stomach but needs to be soluble in the higher pH of the small intestine. We utilized polymer coating

formulas with desired dissolution properties reported in other studies and only adapted the number of coatings accordingly. Capsules were packed with methylene blue powder and dipped in the enteric coating. Coating the capsule in only one, two, or three coatings proved inefficient at resisting degradation in the simulated gastric fluid (SGF) (data not shown). Only until the number of coatings was increased to four total coats did the capsule remain intact for the full duration of the SGF portion of the assay **(Figure 3.2A)**. After 5 hours, the capsule was removed from the SGF and directly placed in the simulated intestinal fluid (SIF). Contrastingly, the coated-capsule rapdily dissolved in SIF, fully dissolving in less than one hour **(Figure 3.2B)**. Four coatings were used for subsequent in vivo studies.

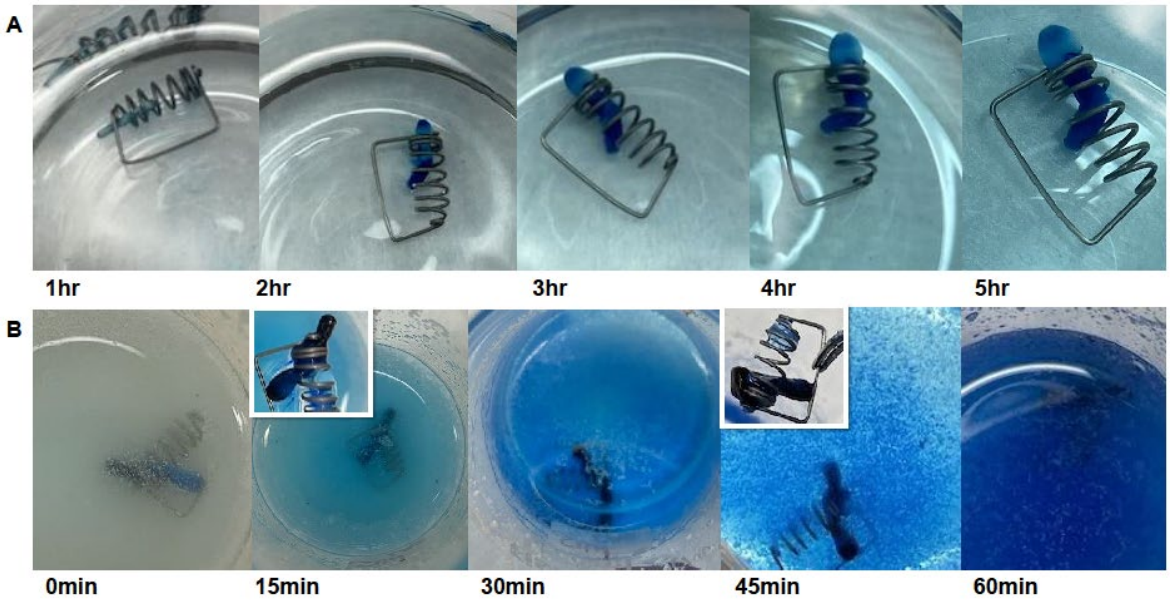


Figure 3.2 In vitro dissolution assay of coated capsules in different pH conditions. (A) Low pH (1.2) dissolution evaluation of capsule over 5hrs, pictures were taken every hour. (B) Same capsule when placed in higher pH (6.8), monitored every 15min for 1hr. (B insert) shows zoomed in pictures of dissolving capsules at 15min and 45min.

3.4.3 Sucrose and HSA Successfully Prevented Lyophilization-Induced Aggregation of VAM

Our vaccine platform is stored as an aqueous solution; thus, we need to make a powder via lyophilization in order to pack it into capsules. The process of freeze drying can cause nanoparticles to increase in size and polydispersity[38]. Human Serum Albumin (HSA) and sucrose were added to VAM vaccine formulations as cryoprotectants.

Dynamic Light Scattering was used to measure the size and polydispersity index (PDI) before and after freeze drying (**Figure 3.3**). HSA (15% w/v) and sucrose (2% w/v) were shown to prevent lyophilization induced aggregation.

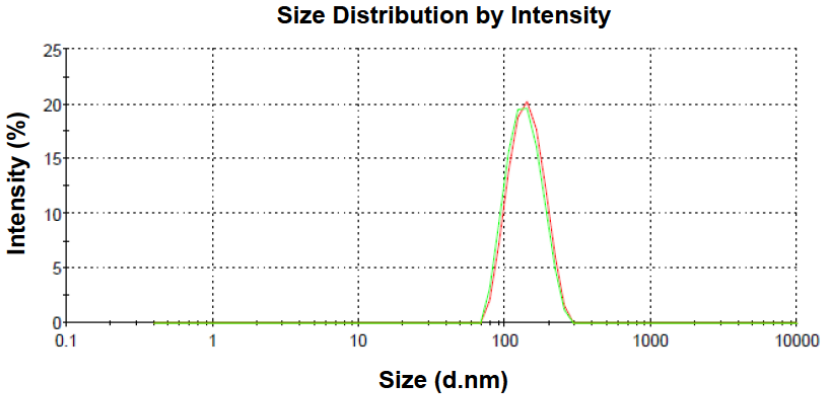


Figure 3.3 Lyophilization of VAM did not induce aggregation. Intensity based size distributions of VAM by Dynamic Light Scattering (DLS) before (green) and after (red) lyophilization.

3.4.4 Capsule Delivery of Lyophilized Vaccine Formulations Failed to Elicit an Immune Response

We wanted to determine if capsule delivery of lyophilized vaccine formulations could enhance mucosal immune responses. To test this, hamsters were dosed orally with one capsule per dose and boosted three times at weekly intervals, 10 days after the last dose feces and sera were collected for antibody determination. Capsules were maximally filled with lyophilized vaccines, containing 200 µg of HER2 peptide and 40 µg of c-di-GMP as adjuvant. As a control, the lyophilized vaccines were reconstituted and administered to hamsters via subcutaneous injection (**Figure 3.4A**). Unfortunately, all groups of hamsters receiving capsule vaccines were negative for antigen-specific IgG antibodies in sera (**Figure 3.4B**). None of the groups had absorbance values higher than the cut off value, which was determined as two times higher than the negative control. The subcutaneous vaccines demonstrated similar serum IgG trends, as shown previously (**Figure 3.4B**). Specifically, the IgG OD450 for VAM was 1.4-fold higher (0.64 ± 0.04 vs 0.50 ± 0.02), 5-fold higher (0.64 ± 0.04 vs 0.36 ± 0.03) than IONP and soluble peptide groups, respectively. Neither SC nor oral gavage vaccine groups were positive for antigen-specific IgA antibodies **(Figure 3.4C).**

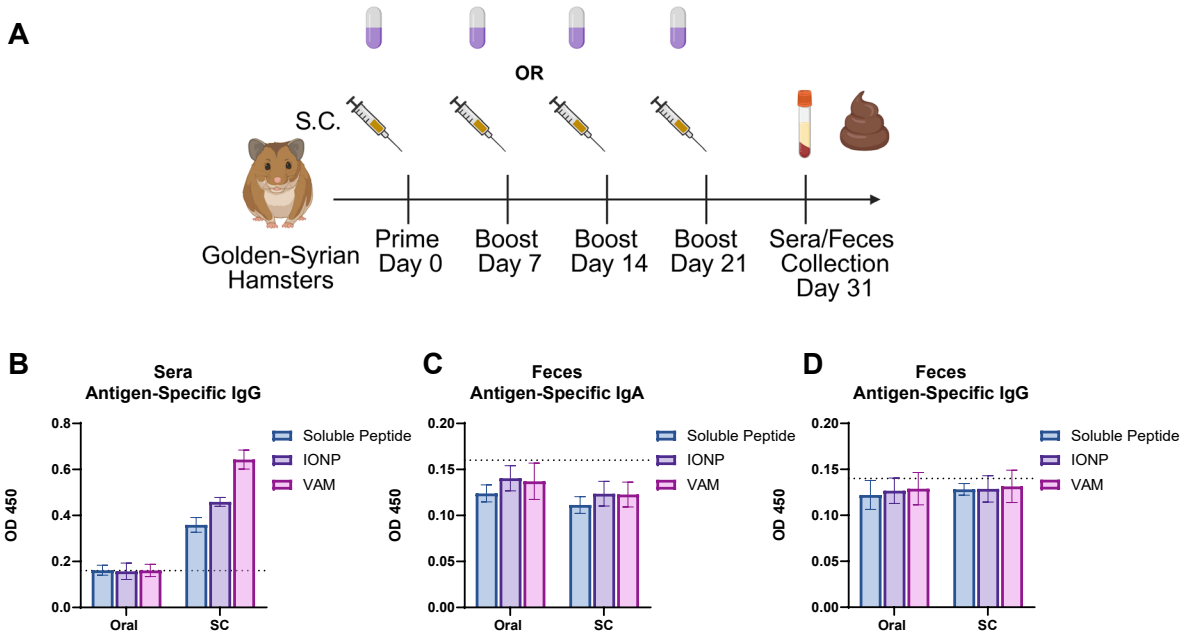


Figure 3.4 Comparing antibody production of vaccines utilizing different delivery strategies (subcutaneous injection vs oral capsule). (A) Animal study immunization and sampling timeline. Antigen- specific antibody OD 450 values after administration of HER2 vaccine; data represent mean ± SD, n = 4. (B) IgG in sera (C) IgA in feces (D) IgG in feces. Dotted lines represent cut-off values, which was calculated from twice the value of the negative control. SC, subcutaneous injection.

3.5 Discussion

Oral delivery of vaccines has many immunological and practical benefits. The dual mucosal/systemic immune response and enhanced patient acceptance make it an attractive method for future vaccine development, especially for vaccines against enteric pathogens[39]. The physiochemical changes across the GI tract make vaccine delivery a challenge.

Delivery of the vaccine to the inductive sites in the small intestine probably remains one of the hardest challenges of an oral vaccine. In addition to numerous pH changes throughout the GI tract, proteolytic enzymes are also present. In our in vitro dissolution study, we only accounted for the pH no enzymes were included in the SGF/SIF. These enzymes could have also contributed to the degradation of the capsule, which might have accelerated its dissolution in the stomach, and prevented its delivery to the small intestine. In future studies, we could repeat our dissolution assay but adding enzymes to the simulated intestinal/gastric fluids and repeat the experiment. We could also perform this experiment in an animal model. By dosing an animal with

our capsule and taking computed tomography (CT) images at set time points, we could track the capsule in real time and have a better understanding of where and when it dissolves.

The Peyer patches, which are responsible for antigen uptake, are primarily localized to the ileum of the small intestine[40]. The ileum is the final section of the small intestine. In our dissolution study, while adding our capsule to the SIF, it dissolved rapidly (within 1hr). In humans the average transit time of food is between 3-5 hours from entry to exit of the small intestine[41]. One study performed an in vivo assay utilizing similar capsules in hamsters. They showed partial degradation of the capsule at 3 hours and completely dissolved within 5 hours, and they were able to detect sIgA after vaccination[37]. Perhaps in our study the capsule dissolved too rapidly in vivo and could not reach the ileum for antigen uptake to occur, thus preventing an immune response from being initiated.

Not only does the capsule need to reach the ileum, but once the capsule dissolves the lyophilized vaccine needs to remain intact for antigen uptake and presentation. Our study did not evaluate the stability of the nanoparticles themselves or vaccine components in SIF. The enzymes and mucus of the GI tract could degrade the surface exposed peptides once it's out of the capsule[42]. Specifically with our VAM platform, we don't know if the gold conjugated to the iron is stable in this type of environment, rendering the benefits of the viral like features useless. After capsule dissolution we do not know the status of VAM, it could be degraded, aggregated, or perfectly stable. Future studies should incubate the nanoparticles themselves in simulated intestinal/ gastric fluid and monitor the nanoparticles over time either by TEM or DLS. We also did not investigate the stability of the gold-peptide bond. Lastly, our vaccine is a subunit vaccine. Subunit vaccines only contain small epitopes of the antigen, so they usually need to be paired with an adjuvant or delivery system to stimulate an immune response[43]. We don't know if the adjuvant is functional after exposure to the GI tract environment. Another study has corroborated the lack of any immune response (mucosal or systemic) following oral administration of c-di-GMP[44]. This further elucidates potential reasons for the lack of antibody production.

A potential downfall of oral vaccines is the need for a bigger dose compared to alternative administration routes. Antigen could get degraded in the stomach and small intestine, so a bigger dose is needed to ensure enough makes it to the inductive sites. For injection vaccines, hamsters typically receive 100-200 μg of purified protein antigen per dose[45]. The 250 μg dose we gave to hamsters might have been too small for an oral vaccine. This doesn't take into account that parental subunit vaccines typically need multiple doses to confer protection[46]. The inadequate dose can also be true for the adjuvant. Ignoring all the delivery issues, even if the vaccine was immunogenic, the antibody production might have been too small to measure.

Despite the numerous shortcomings of our study, we were successful lyophilizing our vaccine, delivering it via subcutaneous injection, and seeing an antibody response. Our previous work did not lyophilize the vaccine prior to administration. This study had similar antibody production trends compared to previous studies, it's safe to assume the lyophilization procedure did not affect the viral-like features of VAM. Many vaccines require storage at 4°C or even lower, maintaining the cold chain until the vaccine can be administered to patients is costly[47]. In future studies, it would be worth investigating lyophilized VAM is stable at room temperature and can retain its immunogenicity.

3.6 Conclusion

Future studies could use this work as a starting point for oral vaccine delivery. The capsule coating could easily be adjusted by adding more coats, ensuring it dissolves slightly slower in the small intestine, so it can make it to the ileum. Even scaling up the dose of antigen and adjuvant wouldn't require much effort. By investigating these two things, we could narrow down the potential issues of our oral vaccine. Even though oral vaccines have their hardships, research should still investigate oral vaccine delivery, the local immune response in the gut would be an advantage in fighting enteric pathogens.

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Chapter 4 Meta Analysis of Cancer Drug Cost Benefit Relationships in FDA-Approved Therapeutics

4.1 Abstract

This study examines the relationship between the rising costs of cancer therapeutics and their clinical benefits as measured by overall survival (OS) and progression-free survival (PFS). We analyzed 214 FDA-approved cancer drugs, including 150 small molecules, 46 antibodies, and 18 antibody-drug conjugates (ADCs), comparing their reported survival benefits against their controls. Our findings reveal a concerning pattern: despite a 50% increase in cancer drug costs over five years, with newly approved treatments averaging $200,000 annually, many provide minimal survival benefits. Among ADCs, 40% failed to improve OS beyond 2.5 months; for antibodies, 36% showed minimal OS benefits and 57% demonstrated limited PFS improvements; small molecules performed slightly better, with approximately 30% providing limited survival benefits. Notably, only a fraction of approved drugs reported both OS and PFS metrics (10/18 ADCs, 20/46 antibodies, 34/150 small molecules), suggesting significant gaps in efficacy documentation. Our analysis highlights the critical need to realign drug development, approval processes, and pricing structures with meaningful clinical outcomes. Value-based pricing models, more stringent approval criteria focusing on patient-centered outcomes, and enhanced post-marketing surveillance may help address the growing disconnect between cancer drug costs and their clinical value. While advances in cancer treatment have contributed to declining mortality rates, the proliferation of high-cost therapeutics with marginal benefits threatens to undermine these gains and exacerbate healthcare disparities.

4.2 Introduction

Cancer is one of the leading causes of death in the United States[1]. The American Cancer Society estimates that in 2025 over 2 million new cancer cases will arise[2]. Overall cancer mortality rates have been declining since the 90s, due to smoking cessation, early detection and improved therapies saving an estimated 4.5 million lives had the rate not declined[3,4]. These gains are threatened by the increase in incidence rates for certain cancers. For instance, the cancer incidence for women younger than 50 has increased almost 30% in the past 20 years[5].

In 2020, the national cost for cancer care in the U.S was estimated to be around $200.7 billion and is estimated to increase to $250 billion by the year 2030[6]. Common chemotherapy drugs can range from $1,000 to more than $12,000 per month[7]. Newly approved cancer drugs can cost an average of $200,000 annually, that's more than a 50% increase in 5 years[8,9]. This financial burden of cancer treatment can influence the treatment decisions of patients, potentially compromising their care[10].

Paradoxically, the 50% increase in treatment costs has not translated to actual significant patient benefits. We will show that many newly approved drugs only increase overall survival (OS) or progression free survival (PFS) by 2.5 months compared to controls.

4.3 Materials and Methods

4.3.1 Collection of OS and PFS Data

We first made a list of cancer drugs with FDA approval labels including 150 small molecules, 46 antibodies (Ab), and 18 antibody drug conjugates (ADC). We looked up the FDA label for each drug and noted the median months for OS and PFS compared to the control. Each graph is organized by drug type (small molecule, Ab, or ADC). For each drug type the median months were plotted for each drug. A second graph was included for each drug type that included the gain, or the difference in median months between the drug and control. For every gain figure, a red dot was included on every drug that was equal to or below 2.5 median months. This was repeated for both OS and PFS. When numbers are listed in the figures the corresponding drug name can be

found in the supplementary information (**Appendix B**). For drugs without PFS or OS the surrogate efficacy evaluation was listed **(Appendix Table B.1- B.3).**

4.4 Results

4.4.1 Antibody Drug Conjugates

Out of the list of 18 antibody drug conjugates only 10 drugs evaluated both OS and PFS. One drug is no longer FDA approved **(Appendix Table B.1**). For OS, the median months for each drug is about 18 months (**Figure 4.1A**). However, when evaluating the gain of these drugs, the median months is much lower. In fact, four out of 10 drugs do not improve overall survival by more than 2.5 months (**Figure 4.1B**). One drug, Datroway, had a gain of less than one month (0.3).

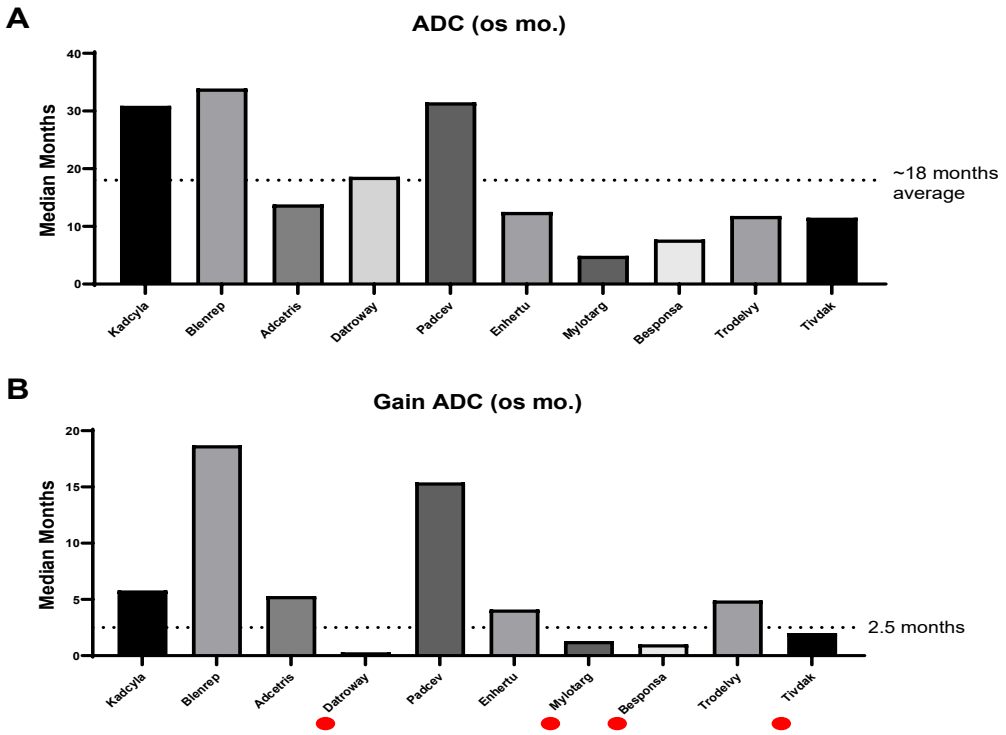


Figure 4.1 Overall Survival of Antibody Drug Conjugates. (A) OS ADC median months. (B) Gain median months overall survival. Red dot denotes less than 2.5 months. ADC: Antibody Drug Conjugate. OS: Overall Survival. Mo: Months

For PFS, the trend is similar. Only 10 drugs from the list evaluated PFS. The PFS was also lower than the OS, with the median months being on average 9 months

(**Figure 4.2A**). Similarly, four out of 10 drugs did not improve progression free survival by more than 2.5 months (**Figure 4.2B**).

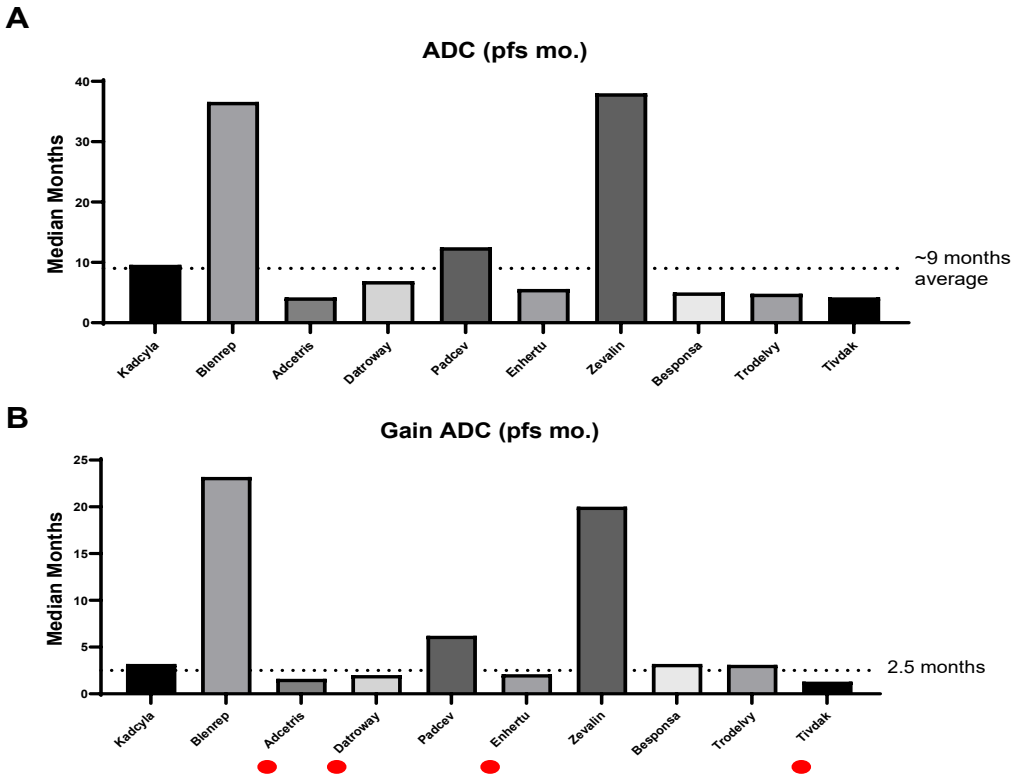


Figure 4.2 Progression Free Survival of Antibody Drug Conjugates. (A) PFS ADC median months. (B) Gain median months Progression Free Survival. Red dot denotes less than 2.5 months. ADC: Antibody Drug Conjugate. PFS: Progression Free Survival. Mo: Months

4.4.2 Antibodies

For antibodies only 20 drugs evaluated both PFS and OS (**Appendix Table B.2**). 22 drugs from the list evaluated OS. The median months for each drug was about 18 months (**Figure 4.3A**). When evaluating the gain, less than 40% (8 drugs) improved overall survival by more than 2.5 months (**Figure 4.3B**). One drug, Margenza, showed a negative gain (-0.3).

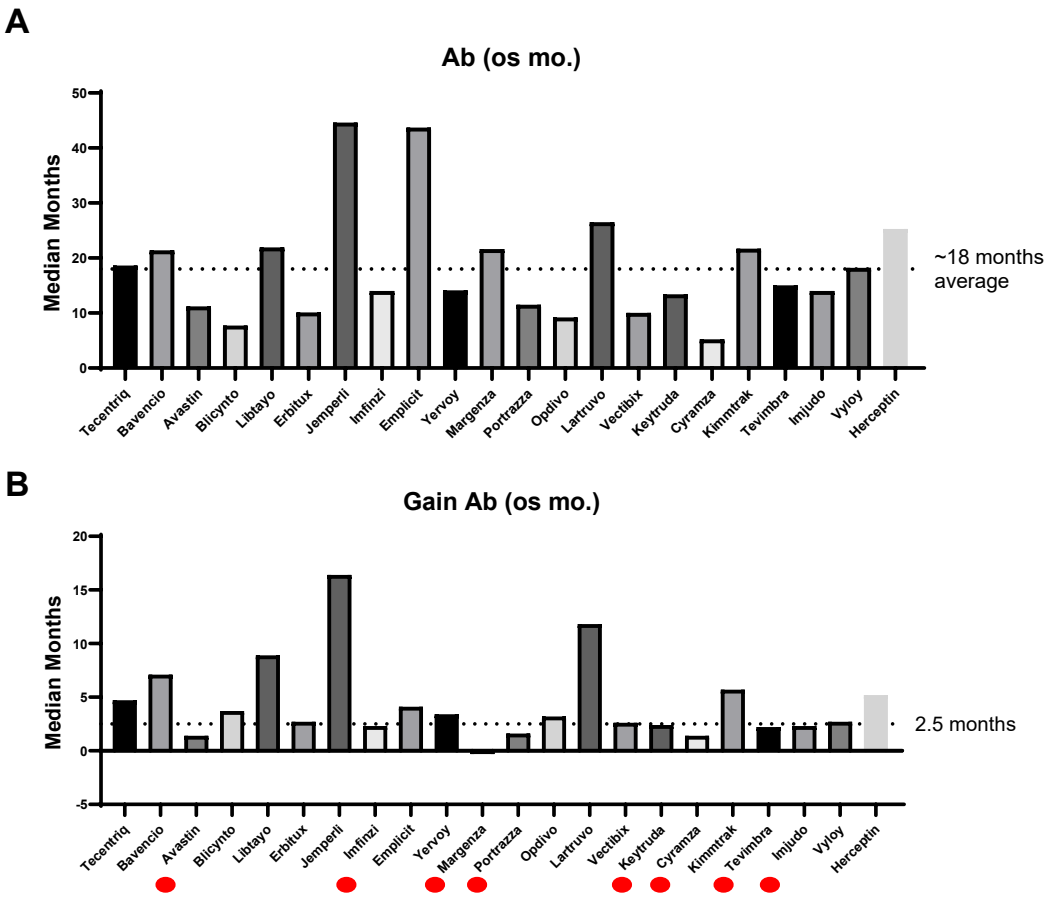


Figure 4.3 Overall Survival of Antibodies. (A) OS Ab median months. (B) Gain median months overall survival. Red dot denotes less than 2.5 months. Ab: Antibodies. OS: Overall Survival. Mo: Months

28 drugs from the list evaluated PFS. The average median months for PFS was about 10 months, again lower than OS (**Figure 4.4A)**. For the gain, more than 50% of drugs failed to improve PFS by more than 2.5 months, specifically 16 drugs (**Figure 4.4B**). Seven drugs even had a gain of less than one month: Tecentriq (0.7), Margenza (0.9), Portrazza (0.2), Opdivo (0.7), Keytruda (0.2), Cyramza (0.8), Kimmtrak (0.4).

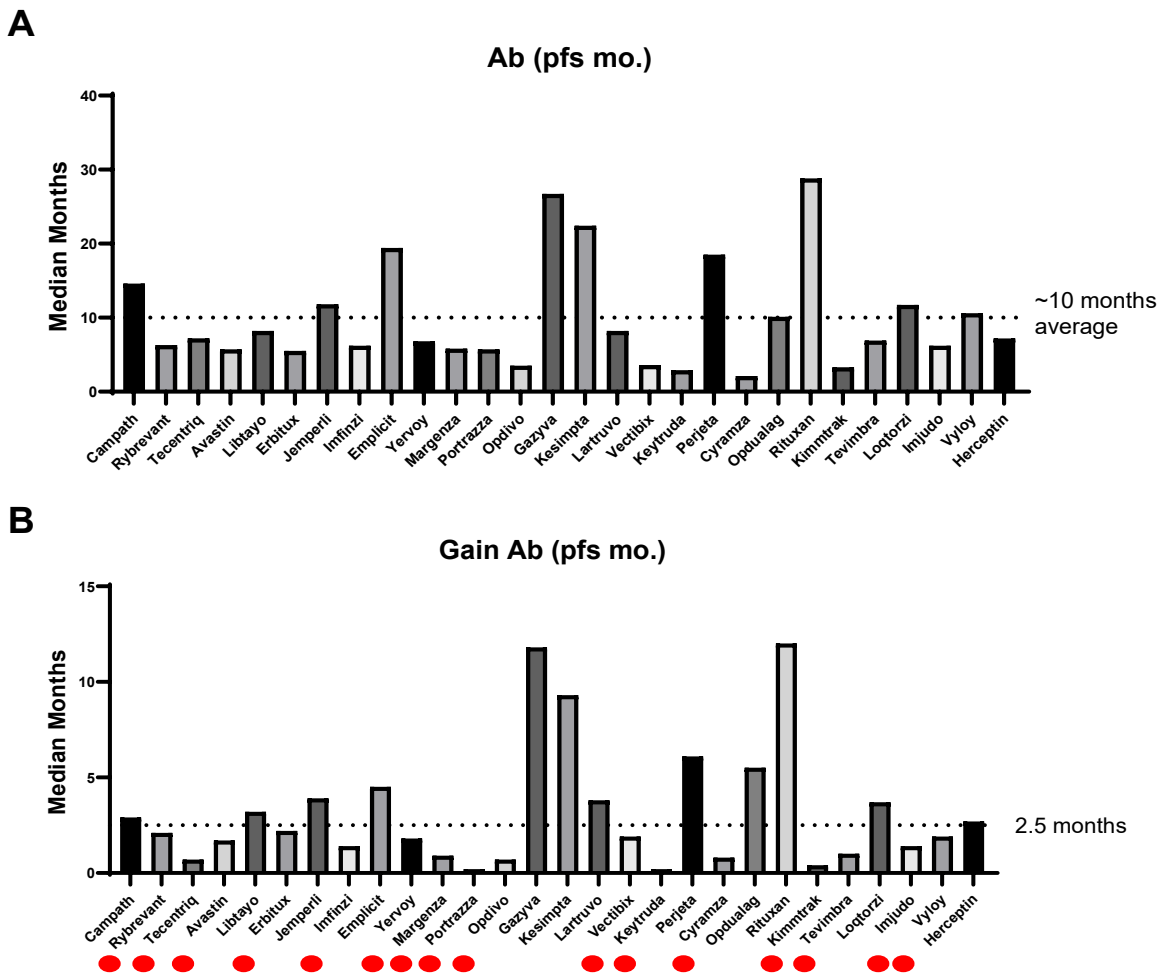


Figure 4.4 Progression Free Survival of Antibodies. (A) PFS Ab median months. (B) Gain median months Progression Free survival. Red dot denotes less than 2.5 months. Ab: Antibody. PFS: Progression Survival. Mo: Months

4.4.3 Small Molecules

For small molecules only 34 of the 150 drugs evaluated both PFS and OS. Four drugs are no longer FDA approved **(Appendix Table B3).** 54 drugs evaluated overall survival. The average median months was 25 months (**Figure 4.5A**). Less than 30%, or 15 drugs, failed to improve overall survival by more than 2.5 months (**Figure 4.5B)**. Two drugs failed to improve gain by more than one month: Doxil (drug 15, 0.7) and Yondelis (drug 50, 0.6). One drug even had a negative gain, Copiktra (drug 16, -3).

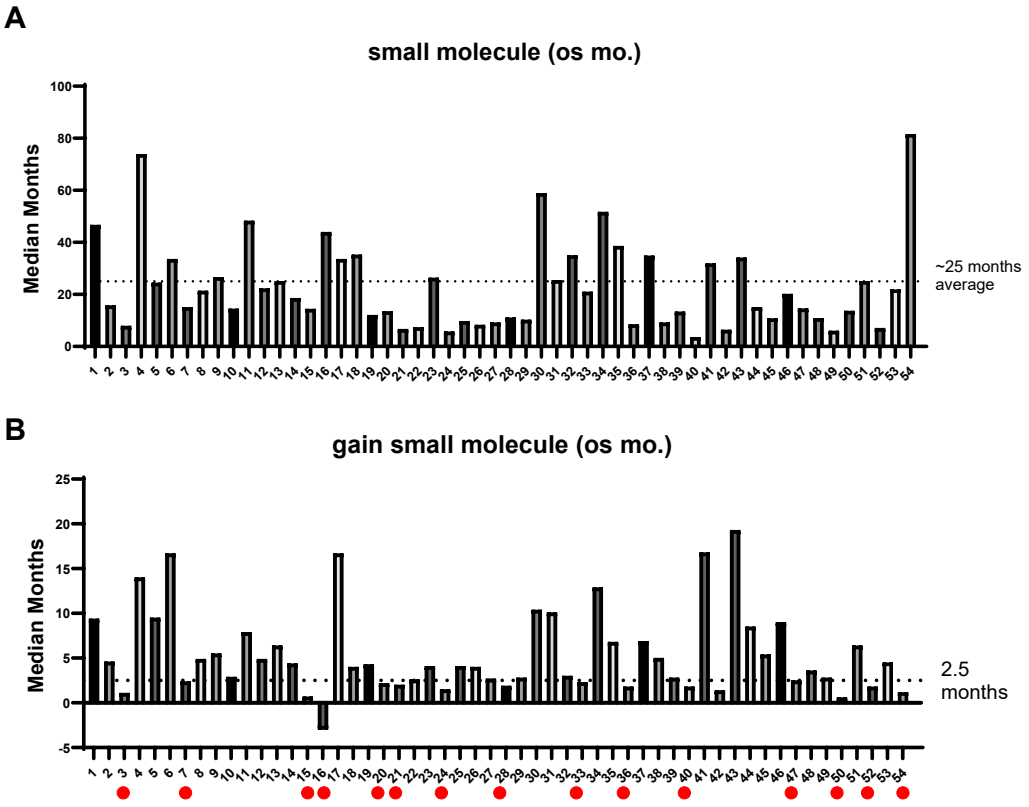


Figure 4.5 Overall Survival of Small Molecules. (A) OS small molecule median months. (B) Gain median months overall survival. Red dot denotes less than 2.5 months. OS: Overall Survival. Mo: Months

For small molecules 70 drugs evaluated progression free survival. The average median months was 13 months (**Figure 4.6A**). Almost 30%, or 22 drugs, failed to improve progression free survival by more than 2.5 months (**Figure 4.6B**). Six drugs failed to improve gain by more than one month: Gilotrif (drug 3, 0.5), Tafinlar (drug 22, 0.5), Tarceva (drug 31, 0.5), Arranon (drug 48, 0.1), Strivarga (drug 57, 0.3), Mekinist (drug 67, 0.5). Two drugs showed no improvement (gain =0): Welireg (drug 8) and Halaven (drug 30).

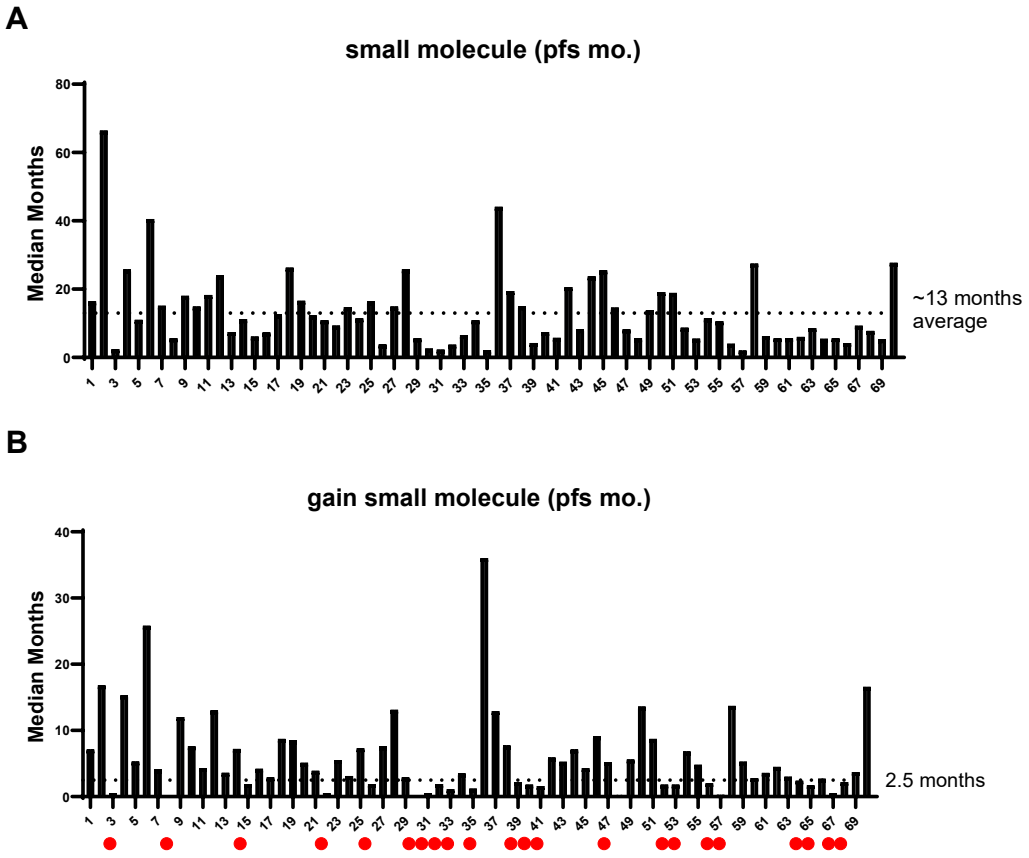


Figure 4.6 Progression Free Survival of Small Molecules. (A) PFS small molecule median months. (B) Gain median months Progression Free survival. Red dot denotes less than 2.5 months. PFS: Progression Survival. Mo: Months

4.5 Discussion

Our comprehensive analysis of FDA-approved cancer therapeutics reveals concerning patterns regarding their clinical benefits relative to their costs. The results demonstrate that a substantial proportion of approved cancer drugs provide marginal survival benefits despite their significant financial burden on healthcare systems and patients. Among the 18 antibody drug conjugates (ADCs) examined, only 10 evaluated both OS and PFS, with one drug no longer maintaining FDA approval. Of those reporting outcomes, 40% failed to improve survival metrics beyond the 2.5-month threshold. 40% failed to improve survival metrics beyond the 2.5-month threshold, with one drug (Datroway) demonstrating a gain of merely 0.3 months in overall survival.

For antibodies, while 46 were initially included, only 20 evaluated both PFS and OS. Among those reporting outcomes, 36% failed to meaningfully improve OS and over 57% showed minimal PFS benefits. Particularly concerning is that one antibody (Margenza) even showed a negative OS gain (-0.3 months), suggesting potential harm rather than benefit. Furthermore, seven antibody drugs demonstrated PFS improvements of less than one month, raising serious questions about their clinical utility despite their high costs.

Small molecules performed marginally better, with approximately 30% of drugs providing limited survival benefits, though notably only 34 of the 150 small molecules evaluated both PFS and OS, with 4 no longer maintaining FDA approval. Within this category, two drugs failed to improve OS by more than one month, and one drug (Copiktra) showed a negative survival impact (-3 months). For PFS, six small molecule drugs demonstrated gains of less than one month, and two drugs (Welireg and Halaven) showed no improvement whatsoever (gain=0).

These findings raise critical questions about the current standards for drug approval and value assessment in oncology. The existence of approved drugs with negative survival impacts or zero benefits is particularly alarming and suggests fundamental flaws in our approval and post-marketing surveillance processes[11,12].

The limited availability of comprehensive efficacy data is concerning. The fact that only a fraction of approved drugs report both OS and PFS metrics (10 of 18 ADCs, 20 of 46 antibodies, and 34 of 150 small molecules) suggests potential gaps in the evidence base supporting cancer drug approvals. This observation aligns with growing concerns about the increasing reliance on surrogate endpoints rather than meaningful survival benefits in the drug approval process[13,14].

The disconnect between drug costs and clinical benefits is particularly troubling. As highlighted in our introduction, the average cost of newly approved cancer drugs has increased by 50% over five years to approximately $200,000 annually, yet this analysis reveals that many of these expensive therapeutics offer minimal survival advantages, with some drugs providing less than one month of additional survival or PFS. This cost-benefit imbalance creates significant financial burden for patients, potentially forcing treatment decisions based on economic rather than clinical factors.

Several factors may contribute to these findings. First, the regulatory pathway for cancer drug approvals has increasingly relied on surrogate endpoints rather than robust OS data, potentially leading to market approval for drugs with limited real-world benefits. Second, the “me-too” drug development approach, where pharmaceutical companies make minor modifications to existing compounds, may contribute to the proliferation of marginally effective therapies[15,16]. Third, the current reimbursement models, which often do not adequately tie payment to performance, provide little incentive for developing truly innovative treatments with substantial survival benefits[17]. The fact that multiple drugs across all three categories have lost FDA approval suggests that post-marketing surveillance has identified issues with some agents, yet many others with minimal benefits remain approved and widely used. This raises questions about whether current post-approval monitoring systems are sufficient to ensure ongoing value, particularly for drugs showing effectively no benefit (gains of 0- 0.3 months)[18].

To address these challenges, we propose several potential solutions. Value-based pricing models that link reimbursement directly to clinical benefit could incentivize the development of more effective therapies[19,20]. Additionally, regulatory bodies should consider implementing more stringent approval criteria that require meaningful improvements in patient-centered outcomes beyond statistical significance[18,21]. Enhanced post-marketing surveillance and reassessment of approved drugs could also help ensure that therapies maintain their value proposition in real-world settings, paying special attention to drugs with minimal or negative impacts[22,23].

Our study has several limitations. We focused solely on OS and PFS as metrics of efficacy, potentially overlooking other important outcomes such as quality of life, toxicity profiles, and treatment convenience. Additionally, our analysis did not account for variations in cancer types, patient populations, or line of therapy, which may influence the interpretation of survival benefits. The incomplete reporting of both OS and PFS across all drug categories also limits comprehensive comparison. Future studies should incorporate these factors to provide a more nuanced understanding of cancer drug value.

4.6 Conclusion

In conclusion, this analysis highlights a critical need to realign the development, approval, and pricing of cancer therapeutics with meaningful patient outcomes. While improvements in cancer care have saved millions of lives, the current trajectory of high-cost, low-benefit drug development threatens to undermine these gains and exacerbate healthcare disparities. A coordinated effort among regulatory agencies, physicians and scientists is essential to ensure that innovations in cancer therapeutics translate to substantive improvements in patient survival and quality of life while maintaining affordability and accessibility.

4.7 Supplementary Information

The supporting information and tables from Chapter 4 are in Appendix B.

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Chapter 5 Conclusion and Future Directions

5.1 Virus Antigen Mimicry Conclusions

This thesis has explored innovative approaches to vaccine development against bacterial pathogens, focusing on both a novel nanoparticle delivery platform and alternative administration routes. The work represents significant advancements in addressing the challenges associated with bacterial vaccine development, particularly for toxin-producing pathogens. Our initial studies demonstrated the successful development of a versatile virus antigen-mimicry nanoparticle vaccine (VAM) platform, combining ultrasmall gold nanoparticles (2-3 nm) with an iron-oxide core (27 nm). This configuration created antigen clusters optimally spaced at 5-10 nm apart, effectively mimicking viral surfaces to enhance B cell receptor binding and promote robust immune responses. The platform demonstrated superior binding to B cell receptors compared to control formulations lacking these viral-like features. Most significantly, in mouse immunization studies, the VAM platform consistently produced antigen-specific IgG titers 3-14-fold higher than control formulations across three distinct bacterial pathogens: *Clostridioides difficile, Bacillus anthracis,* and *Clostridium botulinum*. This versatility is particularly noteworthy, as it suggests the platform's potential applicability to a wide range of bacterial pathogens beyond those tested in our studies. Building upon these promising results, we next explored oral vaccine delivery using our VAM platform to potentially stimulate mucosal immunity. We successfully optimized enteric-coated gelatin capsules that could protect lyophilized VAM from gastric degradation while enabling intestinal release. Through dissolution assays, we determined that four

polymer coatings provided adequate protection in simulated gastric fluid while dissolving rapidly in simulated intestinal fluid. Additionally, we demonstrated that cryoprotectants (15% HSA and 2% sucrose) successfully prevented lyophilization-induced nanoparticle aggregation, maintaining the integrity of the VAM structure and its immunogenic properties when administered subcutaneously. Despite these formulation successes, neither oral gavage of aqueous VAM nor capsule delivery of lyophilized VAM elicited detectable antigen-specific antibody responses in mice and hamsters, respectively. This contrasted with control groups receiving subcutaneous injections, which showed expected immune responses, confirming that the VAM platform maintains its immunogenicity when administered parenterally, even after lyophilization. Several factors may have contributed to the challenges observed with oral delivery. The capsule dissolution kinetics, while promising in vitro, may have led to premature release in the upper small intestine rather than the ileum where Peyer's patches are concentrated. Additionally, the stability of the peptide antigens, gold-peptide bonds, and adjuvants in the gastrointestinal environment was not fully characterized. Furthermore, the antigen dosage used may have been insufficient to overcome the degradation that naturally occurs in the gastrointestinal tract.

5.2 VAM future directions

The promising results from the parenteral administration of our VAM platform suggest several potential applications and directions for future research. The platform's versatility makes it an attractive candidate for developing vaccines against multiple bacterial pathogens, particularly those where traditional approaches have shown limited efficacy. The ability to lyophilize the formulation while maintaining its immunogenicity also presents opportunities for improving vaccine stability and reducing cold chain requirements, which could significantly impact global vaccine distribution and accessibility. Future studies should focus on several key areas.

First, antibody titers (ELISA) only tell part of the story, it does not tell us any information on whether the antibodies actually provide protection again the toxins. Antibodies might not be functional and might not be able to neutralize the toxins. A follow up experiment should be a toxin neutralization assay (TNA). With this in vitro assay, we can determine in the antibodies in sera from vaccinated mice can prevent cell

death/damage from toxin activity. This will give us a better understanding of the toxin neutralization capacity of the antibodies. The TNA again is not conclusive either, we still don't know if our vaccine can prevent serious disease or death. The next set of experiments would involve administering toxin or whole bacteria to vaccinated animals and monitoring their health (weight loss, diarrhea, death, etc. depending on the bacteria). After these experiments we will have a better understanding if VAM provides enhanced protection compared to the control nanoparticles. This can be determined as VAM animals have less death, less severe symptoms, or complete protection meaning no signs of disease. If these experiments are not successful, the next step would be to investigate alternative antigens.

As mentioned, multiple times throughout this thesis, utilizing toxins as the sole antigen demonstrates some efficacy but it does not prevent transmission of the bacteria. We could design a multi-antigen vaccine, that incorporates not only toxin antigens but also antigens to target the bacterium itself. Potential antigens could include the flagella antigen (FLiC), cell wall proteins (CwpV or Cwp84), or surface layer proteins (SlpA), etc. If additional antigens are also unsuccessful then we should adjust our focus to mucosal delivery.

Since our *C. difficile* and *C. botulinum* vaccines aim to prevent colitis or food-borne illness, we could continue to optimize our oral delivery approach. The first experiment could involve keeping everything the same and only increase the dose of the antigen and adjuvant. This would allow us to determine if increasing the dose solves the issue of no antibody production. If increasing the dose does not solve the issue, then more formulation work needs to be completed to ensure intact vaccine delivery to the small intestine.

We could first improve our capsule dissolution assay. We could keep the capsule coating the same and repeat the experiments with added enzymes to see if that changes the dissolution kinetics. We could also perform this experiment in vivo by dosing an animal with a capsule and taking computed tomography scans at designated time points. Both experiments would paint a better picture on how fast the capsule dissolves. The in vivo experiment would also let us tract the capsule its entire journey to the ileum and determine where in the body the capsule dissolves. With this knowledge

we could determine if additional coats need to be applied or if a different polymer coating needs to be used which dissolves at a slightly higher pH. The coating is only one piece of the puzzle, the nanoparticle stability also needs to be verified.

Following capsule dissolution, we need to determine if the nanoparticles themselves are stable in the intestinal environment. Nanoparticles with peptide conjugated should be incubated in intestinal fluid over a set time point and aliquots should be taken at multiple time points. The aliquots can be analyzed by TEM and DLS to determine aggregation, digestion, or if the gold has fallen off VAM. The aliquots can also be centrifuged to pellet the iron, and the supernatant can be analyzed via Liquid Chromatography/ Mass spectrometry (LC/MS). This would allow us to determine if the peptide can also remain intact once the capsule dissolves. We could also investigate if lyophilization caused any changes to our nanoparticles. While we did perform DLS after lyophilization and did not see any size changes, we do not know the status of the peptides. We could perform LC/MS like mentioned earlier and determine if there are any changes to the peptides. All these proposed experiments could help us narrow down the root cause(s) of our vaccines failure to induce antibodies.

For our *B. anthracis* vaccine, we are focused on preventing inhalation anthrax. Since this disease affects the respiratory tract, we could explore intranasal delivery for enhanced stimulation mucosal immunity. A nasal vaccine would have the added benefits of dual mucosal and systemic immunity just like an oral vaccine. However, nasal administration has less physiological and physicochemical barriers to overcome than oral delivery. In conclusion, this work has established a promising nanoparticle vaccine platform that enhances immunogenicity through optimal antigen spacing and presentation. While challenges remain in achieving effective oral delivery, the successful development of the VAM platform represents an important advancement in the field of bacterial vaccine development. The versatility and enhanced immunogenicity of this platform, combined with its potential for lyophilization, could ultimately contribute to more effective vaccines against a range of bacterial pathogens, addressing an important global health challenge.

Appendices

Appendix A: Supporting Information Chapter 2

A.1 Mathematical Modeling

A.1.1 Spheres per g of Fe

The crystalline structure of iron-oxide nanoparticles makes it possible to quantify the number of nanoparticles per unit Fe based on known particle size accordingly to previously reports[1]. Using transmission electron microscopy (TEM), the exact particle size of iron-oxide core (~27nm) was quantified using the ImageJ software. Based on the known unit cell volume of iron-oxide (Fe_3O_4) and quantified particle size, the number of spheres per gram Fe was determined to be 2.55×10^{16} **(Appendix Table A.1)**

Appendix Table A.1 Iron oxide nanoparticle calculations to determine total number of spheres per g Fe.

Description	Value
Unit Cell Volume of Fe_3O_4	0.5905 nm^3
Fe atoms per unit of Fe_3O_4	24
Molecular Weight of Fe	55.85 g/mole
Avogadro's number	6.02×10^{23}
Volume of 27nm IONP Core	10420.93 nm^3
Surface Area of 27nm Sphere	2307.22 nm2
Number of Fe atoms per g Fe	1.08×10^{22}
Number of Fe_3O_4 Unit Cells per g Fe	4.49×10^{20}
Number of Fe_3O_4 per sphere	17647.64
Total Number of Spheres per g Fe	2.55×10^{16}

A.1.2 Peptides per Nanoparticle

The peptide amount per sphere was calculated by first determining the amount of peptide conjugated to the nanoparticles via modified fluorescamine assay (peptide per g Fe). Next, we converted g of peptide to mol of peptide using the average molecular weight of the *C. difficile* peptides. From here we were able to convert to peptide molecules per g Fe using Avogadro's number. Lastly, we were able to determine peptide per sphere by dividing by the previously calculated number of spheres per g Fe. The peptide amount per sphere was determined to be 37,179 and 35,857 for VAM and IONP, respectively **(Appendix Table A.2**). The average was based of three experiments.

Appendix Table A.2 Calculations for peptide per sphere for VAM and IONP.

Description	Value
VAM: Average Amount of Peptide per g Fe	4.3755 g
IONP: Average Amount of Peptide per g Fe	4.22 g
Average Molecular Weight of *C. difficile* Peptides	2752.25
Avogadro's Number	6.02×10^{23}
Total Number of Spheres per g Fe	2.55×10^{16}
VAM: Peptide per Sphere	37,179.19
IONP: Peptide per Sphere	35,857.89

A.1.3 Gold Nanoparticles per μm²

To determine the number of gold nanoparticles (AuNPs) per μm^2, we first needed to determine the amount of AuNPs on VAM. We can do this by extrapolation. The TEM image of VAM was analyzed via ImageJ. A circle was drawn on one VAM particle and the AuNPs in that circle were counted. The diameter of the circle was noted, and the area was calculated. The density of the circle was determined by dividing the gold count by the area. Next, we calculated the surface area of a sphere using VAM's known size. Lastly, to extrapolate the total number of AuNPs on the surface of VAM, we multiplied the density of the circle by the surface area of the sphere. This process was repeated three times on different VAM particles to get an average number of AuNPs. The number of AuNPs per VAM particle was determined to be 92. Once the number of AuNPs was

determined, we could make a final calculation by dividing the AuNPs per VAM particle by the surface area of a sphere and then converting to the proper units. The AuNPs per μm^2 was about 37,500 **(Appendix Table A.3)**

Appendix Table A.3 AuNPs per μm^2.

Description	Value
Average AuNP Count	14.3
Average Circle Diameter	21.97 nm
Average Circle Area	120 nm^2
Average AuNP Density on Circle	0.04 AuNPs/ nm^2
Surface Area of Sphere	2441.6 nm^2
AuNPs per VAM particle	92
Conversion to μm^2	1 x 10^6
Gold per μm^2	37,535

AuNPs, gold nanoparticles

A.2 Bibliography

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Appendix B: Supporting Information Chapter 4

B.1 List of Cancer Drugs Evaluated

B.1.1 Antibody Drug Conjugates (ADCs)

Appendix Table B.1 Antibody Drug Conjugate Evaluations. Median months for Overall Survival (OS) and Progression Free Survival (PFS) of selected Antibody Drug Conjugates (ADCs), or various surrogate efficacy endpoints

	Drug Name (ADC)	OS	PFS	Other
1	ado-trastuzumab emtansine	30.9	9.6	
2	Belantamab mafodotin-blmf	33.9	36.6	
3	Brentuximab Vedotin(Adcetris,Seattle Genetics)	13.8	4.2	
4	Cetuximab saratolacan (Akalux)		Not Approved	
5	datopotamab deruxtecan (Datroway)	18.6	6.9	
6	denileukin diftitox-cxdl (Lymphir,Citius Pharms)			DOR, ORR
7	Disitamab vedotin			DOR, ORR
8	enfortumab vedotin-ejfv	31.5	12.5	
9	fam-trastuzumab deruxtecan-nxki	12.5	5.6	
10	Gemtuzumab ozogamicin (Mylotarg, Wyeth Pharmaceuticals)	4.9		
11	ibritumomab tiuxetan		38	
12	inotuzumab ozogamicin (Besponsa, Pfizer)	7.7	5	
13	Loncastuximab tesirine-lpyl			DOR, ORR
14	mirvetuximab soravtansine-gynx			DOR, ORR
15	Moxetumomab pasudotox		Discontinued	
16	nogapendekin alfa inbakicept-pmln			DOR, CRR
17	Polatuzumab vedotin-piiq (Polivy, Genentech)			DOR, CRR
18	Sacituzumab govitecan (Trodelvy, Immunomedics inc)	11.8	4.8	
19	tisotumab vedotin (Tivdak)	11.5	4.2	

Abbreviations: DOR, Duration of Response; ORR, Overall Response Rate; CRR, Complete Response Rate

B.1.2 Antibodies (Ab)

Appendix Table B.2 Antibody Drug Evaluations. Median months for Overall Survival (OS) and Progression Free Survival (PFS) of selected antibodies, or various surrogate efficacy endpoints

	DRUG NAME	OS	PFS	OTHER
1	alemtuzumab		14.6	
2	amivantamab-vmjw		6.3	
3	atezolizumab (Tecentriq)	18.6	7.2	
4	avelumab (Bavencio, Merck KGaA)	21.4		
5	bevacizumab (Avastin, Genentech)	11.2	5.7	
6	blinatumomab (blicynto)	7.7		
7	cemiplimab-rwlc	21.9	8.2	
8	cetuximab (Erbitux, Imclone)	10.1	5.5	
9	Cosibelimab-ipdl(Unloxcyt, Checkpoint Therapeutics INC)			DOR, ORR
10	daratumumab		NR	DOR, ORR, MRD
11	denosumab			DOR, ORR
12	Dinutuximab(Unituxin,United Therapy)	NR		EFS
13	Dostarlimab-gxly (Jemperli)	44.6	11.8	
14	durvalumab (Imfinzi)	14	6.2	
15	elotuzumab (Emplicit)	43.7		
16	elranatamab-bcmm (Elrexfio)			DOR, ORR
17	epcoritamab-bysp			DOR, ORR
18	glofitamab-gxbm(Columvi,Genentech INC)			DOR, ORR
19	ipilimumab	14.1	6.8	
20	margetuximab-cmkb	21.6	5.8	
21	mosunetuzumab-axgb			DOR, ORR
22	naxitamab-gqgk(Danyelza, Y-Mabs Therapeutics INC)			
23	necitumumab (Portrazza, Eli Lilly)	11.5	5.7	
24	nivolumab (Opdivo, BMS)	9.2	3.5	
25	obinutuzumab (gazyva, Roshe)		26.7	
26	ofatumumab		22.4	
27	olaratumab	26.5	8.2	
28	panitumumab	10	3.6	
29	pembrolizumab	13.4	2.9	
30	pertuzumab		18.5	
31	ramucirumab	5.2	2.1	
32	Relatlimab-rmbw (Opdualag, Bristol Myers Squibb)		10.1	
33	retifanlimab-dlwr (Zynyz, Incyte Corp)			DOR, ORR
34	rituximab		28.8	
35	tafasitamab-cxix (Monjuvi, Morphosys US INC)			ORR
36	talquetamab-tgvs			DOR, ORR

37	tarlatamab-dlle (Imdelltra, Amgen INC)			DOR, ORR
38	tebentafusp-tebn (Kimmtrak, Immunocore LTD)	21.7	3.3	
39	teclistamab-cqyv (Tecvayli, Janssen Biotech)			DOR, ORR
40	tislelizumab-jsgr (Tevimbra)	15	6.9	
41	Toripalimab-tpzi		11.7	
42	tremelimumab	14	6.2	
43	Zanidatamab			DOR, ORR
44	zolbetuximab-clzb (Vyloy)	18.2	10.6	
45	zenocutuzumab-zbco (Bizengri, Merus NV)			DOR, ORR
46	trasuzumab	25.3	7.2	

Abbreviations: DOR, Duration of Response; ORR, Overall Response Rate; NR, Not Reached; MRD Measurable Residual Disease; EFS, Event Free Survival

B.1.3 Small Molecules

Appendix Table B.3 Small Molecule Evaluations. Median months for Overall Survival (OS) and Progression Free Survival (PFS) of selected small molecules, or various surrogate efficacy endpoints

	Drug	OS	PFS	Other
1	abemaciclib (Verzenio, Eli Lilly)	46.7	16.4	
2	abiraterone acetate (Zytiga, Jassen Pharmaceuticals, subsidiary of J &J)	15.8		
3	acalabrutinib (Calquence, Astrazeneca)		66.4	
4	adagrasib (Krazati, Bristol)			DOR, ORR
5	afatinib dimaleate	7.9	2.4	
6	alectinib		25.7	
7	alpelisib (Piqray, Novartis)		11	
8	alpelisib (Vijoice, Novartis)			DOR, ORR
9	anastrozole (Arimidex, ANI Pharms)			DFS, DOR
10	apalutamide (Erleada, Janssen Biotech)	73.9	40.5	
11	asciminib(Scemblix, Novartis)			MMR
12	avapritinib			DOR, ORR
13	axitinib		15.1	
14	azacitidine (Vidaza, Bristol-Myers)	24.5		
15	belinostat (Beleodaq, Acrotech Biopharma)			DOR
16	belzutifan (Welireg, Merck Sharp&Dohme)		5.6	
17	bendamustine hydrochloride (Bendeka, Eagle Pharms)		18	
18	binimetinib (Mektovi, Array Biopharma INC)	33.6	14.9	
19	Bortezomib (Velcade, J&J)		18.3	
20	bosutinib			MMR
21	brigatinib(Alunbrig,Takeda)		24	
22	cabazitaxel(Jevtana, Sanofi)	15.1		

23	Cabozantinib (Cabometyx, Exelixis) (Cabozantinib S-Malate)	21.4	7.4	
24	Cabozantinib (Cometriq, Exelixis)	26.6	11.2	
25	capecitabine (Xeloda, Chepla Pharm)	14.5	6.1	
26	Capivasertib		7.3	
27	Capmatinib (Tabrecta, Novartis)		12.6	
28	carfilzomib	48.3	26.3	
29	Ceritinib (Zykadia, Novartis)		16.6	
30	clofarabine			CR
31	cobimetinib (Cotellic, Genentech)	22.3	12.3	
32	copanlisib(Aliqopa, Bayer Healthcare)		withdrawn	
33	crizotinib		10.9	
34	dabrafenib	25.1	9.3	
35	Dacomitinib (Pfizer, Vizimpro)		14.7	
36	darolutamide	NR		MFS
37	dasatinib			MMR
38	Dectabine (Dacogen, Janssen-Cilag & Otsuka)			DOR, ORR
39	degarelix (Firmagon, Ferring)			castration rates
40	docetaxel	18.6	11.4	
41	doxorubicin hydrochloride (Adriamycin, Pfizer)	NA		DFS
42	doxorubicin hydrochloride liposomal injection (Doxil, Sun Pharm)	14.4		
43	duvelisib (Copiktra, Secura)	44	16.4	
44	elacestrant (Orserdu, Stemline Therapy)		3.8	
45	enasidenib (Idhifa, Bristol Myers Squibb)			CR
46	encorafenib (Braftovi, Array BioPharma)	33.6	14.9	
47	ensartinib (Ensacove, Xcovery)		25.8	
48	entrectinib			DOR, ORR
49	enzalutamide (Xtandi, Astellas)	35.3		
50	erdafitinib (Janssen Biotech, Balvers)	12.1	5.6	
51	eribulin (Halaven, Eisai Inc)	13.5	2.6	
52	Erlotinib (Tarceva, Roche)	6.7	2.3	
53	everolimus (Zortress, Novartis)	prophylactic organ rejection		
54	Exemestane (Aromasin, Pfizer Inc.)			DOR, ORR
55	fedratinib (Inrebic, Bristol-Myers)			spleen vol
57	fruquintinib (Fruzaqla, Chi-Med)	7.4	3.7	
58	fulvestrant	26.4	6.5	
59	Futibatinib (Lytgobi, Taiho Oncology			DOR, ORR
60	gefitinib		10.9	
61	gemcitabine	5.7	2.1	
62	gilteritinib(Xospata, Astellas Pharma)	9.7		
63	glasdegib(Daurismo, Pfizer)	8.3		
64	ibrutinib		44.1	

65	idelalisib (Zydelig, Gilead)		19.4	
66	imatinib mesylate (Gleevec, Novartis)		NA	
67	inavolisib (Itovebi, Genentech)		15	
68	infigratinib (Truseltiq, QED Therapeutics)		withdrawn	
69	irinotecan hydrochloride	9.2	4.2	
70	irinotecan liposome injection (Onivyde, Ipsen Biopharmaceuticals)	11.1	7.4	
71	ivosidenib	10.3		
72	ixabepilone		5.7	
73	ixazomib(Ninlaro, Takeda Pharms USA)		20.6	
75	lanreotide		NR	
76	lapatinib ditosylate		8.26	
77	larotrectinib			ORR
78	Lazertinib (Lazcluze, Janssen biotech)		23.7	
79	lenalidomide	58.9	25.5	
80	Lenvatinib (Lenvima, Eisai & Co)	25.5	14.6	
81	letrozole	35		
82	lorlatinib			DOR, ORR
83	lurbinectedin (Zepzelca, Jazz)			DOR, ORR
84	melphalan flufenamide		withdrawn	
85	midostaurin (Rydapt, Novartis)		8.2	
86	Mitomycin (Jelmyto, Urogen)			CR
87	mobocertinib (Exkivity, Takeda)			DOR, ORR
88	Momelotinib			spleen vol
89	Nelarabine	21	5.6	
90	Nilotinib			MMR
91	Niraparib		13.8	
92	Nirogacestat (Ogsiveo)		NR	ORR
93	Olaparib (Lynparza, Astra Zeneca)	51.7	19.1	
94	Olutasidenib (rezlidhia, Rigel PHARMS)			CR
95	omacetaxine mepesuccinate (Synribo, Teva)			MCyR
96	osimertinib (Tagrisso, Astrazeneca)	38.6	18.9	
97	oxaliplatin (Eloxatin, Pfizer)		8.7	
98	paclitaxel protein-bound particles for injectable suspension (abraxane, Celgene)	8.5	5.5	
99	pacritinib(Vonjo, Sobi)			spleen vol
100	pafolacianine	Imaging agent		
101	palbociclib	34.9	11.4	
102	panobinostat		10.6	
103	pazopanib hydrochloride	9.2		
104	Pelmetrexed (Alimta, Lilly)	13.4	4	
105	pemigatinib (Pemazyre, Incyte)			DOR, ORR
106	pexidartinib			DOR, ORR

107	pirtobrutinib			DOR, ORR
108	pomalidomide	3.6		
109	ponatinib (Iclusig)			MCyR
110	pralatrexateb injection (Folotyn)			DOR, ORR
111	pralsetinib (Gavreto)			DOR, ORR
112	quizartinib (Vanflyta)	31.9		
113	regorafenib (Stivarga)	6.4	2	
114	relugolix (Orgovyx)			castration rates
115	Repotrectinib (Augtyro)	34.1		
116	Revumenib(revuforj,Syndax)			CR
117	ribociclib		27.5	
118	Ripretinib(Qinlock,Deciphera PHARMS)	15.1	6.3	
119	romidepsin (istodax)			DOR, ORR
120	rucaparib (Rubraca)	10.8		
121	ruxolitinib (Jakafi/Jakavi)			spleen vol
122	selinexor (Xpovio)			ORR
123	selpercatinib (Retevmo)	20.2		
124	selumetinib (Koselugo)			DOR, ORR
125	sirolimus protein-bound particles for injectable suspension (albumin-bound), (Fyarro, Aadi Bioscience Inc)			DOR, ORR
126	sonidegib			DOR, ORR
127	sorafenib tosylate (Nexavar, Bayer)		5.6	
128	sotorasib		5.6	
129	sunitinib malate (Sutent, Pfizer)		6	
130	talazoparib (Talzenna, Pfizer)		8.6	
131	tazemetostat (Tazverik, Epizyme Inc)			DOR, ORR
132	temozolomide	14.6		
133	temsirolimus (Torisel, Pfizer)	10.9	5.5	
134	tepotinib hydrochloride			DOR, ORR
135	thalidomide (Thalomid, Bristol Myers Squibb)			ORR
136	tivozanib (Fotivda, AVEO Oncology)		5.6	
137	topotecan (Hycamtin, Novartis)	6.0		
138	Tovorafenib (Ojemda, Day One BioPharms)		NR	ORR
139	trabectedin (Yondelis, J & J)	13.7	4.2	
140	TRAMETINIB(Mekinist,Novartis)	25.1	9.3	
141	trifluridine, tipiracil	7.1		
142	tucatinib (Tukysa, SeaGen)	21.9	7.8	
143	umbralisib		withdrawn	
144	Vandetinib (Caprelsa, Astrazeneca)	81.6		
145	vemurafenib (Zelboraf, Genentech)		5.3	
146	venetoclax (Venclexta, Abbvie)		NR	
147	vismodegib			ORR

148	Vorasidenib（Voranigo,Servier）	27.7	
149	vorinostat(Zolinza,MSD sub Merck)		CCR, PR
150	zanubrutinib (Brukinsa, BeiGene)		DOR, ORR

Abbreviations: DOR, Duration of Response; ORR, Overall Response Rate; DFS, Disease Free Survival; MMR, Major Molecular Remission; CR, Complete Remission; MFS, Metastasis-Free Survival; Spleen Vol, Spleen Volume; MCyR, Major Cytogenetic Response; PR, Partial Remission; NR, Not Reached; CCR, Complete Cytogenetic Response