Chapter 11

Defense Against Biological Terrorism: Vaccines and Their Characterizations

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Abstract This chapter presents an introduction to infective diseases and potential biological agents that could potentially be used for bioterrorism. Technical brief description of the possible treatments and preventions of these biothreats is presented with emphasis placed on the principles of immunological defenses, vaccination, and preparation of vaccines. In principle, various types of vaccines that are commercially used as “antibacterial or anticancer vaccines” can be produced using various types of antigenic carbohydrate haptens containing relevant epitopes. This review highlights the strategies used for the characterization of such synthetic neoglycoconjugate vaccines used as a means of protection against biothreats. A complete mass spectrometry-based strategy for validating the preparations of the neoglycoconjugate vaccine is presented.

Keywords Biological agents · Toxins · Poisons · Immunology · Vaccines · Neoglycoconjugates · Mass spectrometry
11.1 Classification of Diseases Caused by Biological Terrorism

In the light of recent concern and interest about the potential for biological terrorism (biowarfare), there are several diseases and bacterial toxins that must be considered in particular, like anthrax [1, 2], smallpox [3, 4], plague [5], botulinum toxin [6], and tularemia [7]. A very detailed discussion of such diseases and other infectious diseases with similar risks in terms of bioterrorism goes beyond the scope of this concise chapter, but some features of these and other infectious diseases representing important threats in the biowarfare field will be mentioned. In this respect, we may distinguish in time diseases which are:

1. old diseases which are disappearing and sometimes returning, like smallpox and poliovirus infections (which are either extinct or close to be eradicated, thanks to planetary vaccination programs);
2. diseases still active at present times, like carbuncle (anthrax), plague, tularemia, tetanus, botulinum, TBC, etc.;
3. New diseases, which are appearing/spreading, like SARS (Severe Acute Respiratory Syndrome) and its more recent variety of MERS (Middle-East Respiratory Syndrome), infections by Ebola/Marburg viruses, hantavirus, filovirus, novel Flu virus strains and coronavirus COVID-19.

The next section is dedicated to the essential facts concerning these diseases. For a complete medical reference to all of them, see, for instance, the Merck Manual of Diagnosis and Therapy [8].

11.1.1 Old Infective Diseases

11.1.1.1 Smallpox (Variola)

Smallpox is a highly contagious disease (incubation 10–12 days) caused by the smallpox virus, an orthopoxvirus. It causes death in up to 30% of infected subjects. The indigenous infection has been eradicated (last case, Ethiopia, 1990 – WHO). The main concern for outbreaks of smallpox is today from bioterrorism. Smallpox is characterized by severe constitutional symptoms (fever, headache, extreme malaise) and a characteristic pustular rash. Treatment is supportive; prevention involves vaccination, which, because of its risks (eczema, encephalitis, etc.), is done selectively.

The pathogenesis of smallpox demonstrates that the virus is transmitted from person to person by direct contact or inhalation of droplet nuclei. Clothing and bed linens can also spread infection. Most contagions are in the first 7–10 days after the skin rash appears. Once crusts form, the infectivity declines. The virus invades the oropharyngeal and respiratory mucosa, multiplies in regional lymph nodes, causing viremia and localization in small blood vessels of the skin (rash) and rarely in CNS (encephalitis).
Officially, smallpox has been wiped out in the world. There are no longer cases detected in the world population since 1990. An ethical question remains concerning smallpox. Are we allowed to destroy old samples of smallpox virus (used as standard reference material), which are kept in some virology laboratories around the world? Certainly not [3], because we could no longer prepare proper vaccines without the live virus samples to start from. And without the vaccine, a small number of wild viruses could ignite a wide epidemic killing a large proportion of the human population since the vaccination is no longer mandatory in any country, and a large percentage of young populations have no longer been vaccinated after the early 1990s.

### 11.1.2 Existing Active Infective Diseases

#### 11.1.2.1 Anthrax (Carbuncle)

Anthrax is caused by *Bacillus anthracis*, toxin-producing, encapsulated, aerobic or facultative anaerobic organisms. Anthrax, an often-fatal disease of animals, is transmitted to humans by contact with infected animals or their products (woolsorter’s disease). In humans, infection typically occurs through the skin. Inhalation infection is less common; oropharyngeal, meningeal, and GI infections are rare. For inhalation and GI infections, nonspecific local symptoms are typically followed in several days by severe systemic illness, shock, and often death. Empiric treatment is with ciprofloxacin or doxycycline. A vaccine is available (antitoxin).

Pathogenesis of anthrax takes place since *Bacillus anthracis* readily forms spores when germs encounter a dry environment—a condition unfavorable for growth. Spores resist destruction and can remain viable in soil, wool, and animal hair for decades.

Spores germinate and multiply in favorable conditions (wet skin, tissue, blood) and can give human disease by contact (papules, black eschars, contagious also via fomites), ingestion (raw meat → fever, nausea, vomiting, diarrhea), and inhalation (flu-like illness, respiratory distress, cyanosis, shock, coma). Needless to say, that biothreats with anthrax attacks through mailings (using spores in powder form) have already taken place in the USA in 2001 (US Postal Service, Washington DC). This event highly sensitized the public to the global theme of bioterroristic attacks.

#### 11.1.2.2 Plague (Pestis, Black Death)

Plague is caused by *Yersinia pestis* (formerly named *Pasteurella pestis*). Short bacillus with hairpin shape, infects wild rodents and can infect humans via tick bites. Symptoms are either severe pneumonia or massive lymphadenopathy with high fever, often progressing to septicemia. Diagnosis is epidemiologic and clinical, confirmed by culture and serologic testing. Treatment is with streptomycin or doxycycline. Unfortunately, a vaccine is not available for the plague.
11.1.2.3  Tetanus

Tetanus is an acute poisoning from a neurotoxin produced by Clostridium tetani. Symptoms are intermittent tonic spasms of voluntary muscles. The spasm of the masseters accounts for the name “lockjaw” (trismus). Incubation requires 2–10 days. The diagnosis is clinical. Treatment is with immune globulin and intensive support. The only unbound toxin can be neutralized. A vaccine is available, with a good extent of preventive protection.

11.1.3  New Infective Diseases

11.1.3.1  Ebola/Marburg Diseases

Marburg and Ebola are filoviruses that cause hemorrhage, multiple organ failure, and high mortality rates. Diagnosis is with enzyme-linked immunosorbent assay, PCR, or electron microscopy. Treatment is supportive. Strict isolation and quarantine measures are necessary to contain outbreaks. Incubation 5–10 days. Marburg virus has been identified in bats and primates. Human to human transmission occurs via skin and mucous membranes contact (humans/primates). Filoviruses can affect intestines (nausea, vomiting, diarrhea), respiratory tract (cough, pharyngitis), liver (jaundice), CNS (delirium, stupor, coma), and cause hemorrhagic phenomena (petechiae, frank bleeding) with high mortality rates (up to 90% with Ebola virus). Survivors recover very slowly and may develop long-lasting complications (hepatitis, uveitis, orchitis) with only supportive care available. No specific antivirals nor vaccines are available for filovirus infections.

11.1.3.2  Influenza Virus, with New Strains, Continuously Appearing

Last but not least, we must now mention influenza! Flu viruses are in nature among the most rapidly changing (mutating) organisms through their ability to infect a variety of hosts: birds (migrating waterfowl -ducks-, stantial poultry -chickens-), mammals (pigs, felines), and humans. In southeast Asia (mostly in China, but also in Viet-Nam, Laos, Thailand, etc.), it is widespread to have mixed farms of pigs, poultry and ducks, attended by humans. Every year, new strains appear in SE-Asia is favored by the reciprocal passage between migrating birds (mostly fowl), pigs, and chickens, with exposure of many humans in farms, markets, rooster fighting sports, and food preparation places.

A common say in China tells that “Anything with four legs (except chairs), and anything that flies (except airplanes) can be eaten.” With this philosophy, there is generally a lot to be desired in food safety and general hygienic prevention in such geographical areas.
After the avian flu H5N1 of 2005–2006, highly lethal but unable to give human to human contagion, new combinations of flu strains are expected and feared, with high lethality and high human to human transmissibility.

### 11.1.4 New Virus Causing World Pandemic

Actually, concerning the world diffusion of new virus strains with pandemic potential, the world has been shaken by the recent coronavirus, also known as COVID-19, which has spread to all countries in the world. A new protocol to investigate the extent of COVID-19 infection in the population, as determined by positive antibody tests in the general population, has been developed. The protocol is titled the Population-based age-stratified seroepidemiological investigation protocol for COVID-19 virus infection.

### 11.1.5 General Considerations

In summary, we can see that continuous worldwide biomedical surveillance caused by perceived biotreats or actually new infective agents, is a primordial task that needs to be maintained and improved. For this reason, it is imperative that the worldwide appearance of new strains of viruses, like COVID-19, demands immediate and analytical precise isolation and characterization of the new viruses. This is required in order to isolate as soon as possible the potentially pandemic new strains and to prepare biological stocks suitable for massive vaccine preparations in due time to prevent the global spreading of potentially lethal new viruses. Examples in time recall the cases of the highly lethal pandemics known as “Spanish flu” in 1917–1918 (more than 40 million deaths worldwide), “Asian flu” in 1956 (over 100,000 deaths worldwide), “Hong Kong flu” in 1978 (in excess of 700,000 deaths worldwide) and finally COVID-19 [9].

Recently, a novel SARS-CoV-2 virus, also known as the COVID-19 virus, has shown to be highly contagious, pathogenic, and rapidly spreading. This novel SARS-CoV-2 has caused a global pandemic COVID-19, which has severely affected the health and economy of several countries. It was found that this novel COVID-19 virus can enter the host cell, by the specific binding resulting from the viral surface spike glycoprotein (S-protein) to the converting angiotensin enzyme 2 (ACE2). This specific virus molecular interaction with the host cell represents a promising therapeutic throughput virtual screening approach that was used to target for identifying SARS-CoV-2 (COVID-19) antiviral drugs.

To sum it up, the primary screening of any biological agents capable of causing a world pandemic needs to be identified as soon as possible, in order to find the promising drugs or immunotherapeutic agents.
11.2 Biothreats and Risk Level Assessment

The ability to identify counter bioterrorism depends on the information generated by researchers on the disease-causing microorganisms and also on understanding the immune system [10, 11]. It was shown that biothreat caused by biological agent weapons could be classified on the following four criteria:

(i) the impending treat to cause morbidity and mortality in healthy individuals;
(ii) the potential of the biological agent to spread within the community;
(iii) the potential to elicit fear or panic alleged by the biological agent threat and;
(iv) the capability of public health responders to counteract and control the biothreat

In response to the possible biological threat, a list of selected biological agents threat named the “Priority Pathogens” list was created for countermeasures, including vaccines. A complete list of these agents with their respective designations (Category A–C) is presented in Table 11.1 [10].

11.2.1 Category A Agents

The Category A biological agents comprise four highly infectious pathogens that can be transmitted as aerosols. Consequently, Bacillus anthracis, Yersinia pestis, smallpox (variola major), and Francisella tularensis were contemplated as being of the greatest threat to public safety, because of their highly pathogenic nature, ability to spread from person-to-person [12]. In addition, botulinum neurotoxin (BoNT), which can be aerosolized, belongs to Category A Agents, as it is one of the most lethal known biological toxins [13]. It should be repeated that smallpox and anthrax are the biothreats that pose the greatest risk for causing large numbers of casualties in the event of an effective release by a terrorist group.

11.2.2 Category B Agents

The Category B biological agents are known to have the capability to moderately disseminate, and are capable of inflicting moderate morbidity/low mortality. Category B biological agents require specific improvements to the known standard diagnostic capacity. The majority of the Category B agents attack the gastrointestinal tract and are introduced in the human system by food and water ingestion. Such Category B agents that pose safety threats include the Salmonella and Shigella species, pathogenic Vibrios, enterotoxigenic E. coli, as well as toxins such as ricin, staphylococcal enterotoxin B. In general, the Category B agents are not communicable from person to person but are rather easy to disperse to cause highly debilitating sicknesses that need immediate medical attention.
### Table 11.1 Category A–C Bio threats. Copied from reference [10]

| Toxins                          | Central European tick-borne encephalitis<sup>a</sup> |
|---------------------------------|------------------------------------------------------|
| Botulinum neurotoxins<sup>a,A</sup> |                                                   |
| Shigatoxin<sup>a</sup>          |                                                     |
| Tetrodotoxin<sup>a</sup>        |                                                     |
| T-2 toxin<sup>a</sup>           |                                                     |
| Staphylococcal enterotoxins<sup>a,B</sup> |                                             |
| Ricin<sup>a,A</sup>             |                                                     |
| Conotoxins<sup>a</sup>          |                                                     |
| Abrin<sup>a</sup>               | Severe acute respiratory syndrome (SARS)<sup>C</sup> |
| Saxitoxin<sup>a</sup>           |                                                     |
| Shiga-like ribosome inactivating proteins<sup>a</sup> |                                               |
| Clostridium perfringens epsilon toxin<sup>B</sup> | West Nile virus<sup>B</sup>                           |
| **Virus**                      |                                                      |
| Eastern equine encephalitis<sup>a,B</sup> | Bacteri/a/rickettsia                                 |
| Hendra virus<sup>b</sup>        | Bacillus anthracis<sup>b,A</sup>                   |
| Variola major (smallpox)<sup>a,A</sup> | Brucella abortus<sup>b</sup>B                  |
| Variola minor (alastrim)<sup>a</sup> | Brucella melitensis<sup>b,B</sup>                |
| Monkeypox<sup>a</sup>           | Brucella suis<sup>b</sup>B                          |
| Filoviruses                    |                                                    |
| Ebola virus<sup>a,A</sup>       | Burkholderia mallei<sup>P,B</sup>                  |
| Marburg virus<sup>a,A</sup>     | Burkholderia pseudomallei<sup>P,B</sup>           |
| Arenaviruses                    | Francisella tularensis<sup>A,A</sup>              |
| Junin<sup>a,A</sup>             | Yersinia pestis<sup>a</sup>A                        |
| Machupo<sup>a</sup>             | Rickettsia prowazekii<sup>a,B</sup>               |
| Guanarito<sup>a,A</sup>         | Rickettsia rickettsii<sup>a</sup>                 |
| Flexal<sup>a</sup>              | Pathogenic vibrios<sup>B</sup>                     |
| Sabia<sup>a,A</sup>             | Shigella species<sup>B</sup>                       |
| Lassa<sup>a,A</sup>             | Salmonella species<sup>B</sup>                     |
| Japanese encephalitis virus<sup>B</sup> | Listeria monocytogenes<sup>B</sup>                 |
| Venezuelan equine encephalitis<sup>a,B</sup> | Yersinia enterolitica<sup>a</sup>               |
| Dengue<sup>A</sup>              | Campylobacter jejuni<sup>B</sup>                  |
| LaCrosse<sup>B</sup>            | Multi-drug resistant tuberculosis<sup>C</sup>     |
| California encephalitis<sup>B</sup> | Other Rickettsia<sup>C</sup>                     |
| Western equine encephalitis<sup>B</sup> | Chlamydia psittaci<sup>A</sup>              |
| Bunyaviruses                    | Diarrheagenic E. coli<sup>B</sup>                 |
| Hantaviruses<sup>A</sup>        | Botulinum toxin-producing species of Clostridium<sup>a</sup> |
| Rift Valley Fever<sup>p,A</sup>| Protozoa                                            |
| Chikungunya<sup>C</sup>        | Cryptosporidium parvum<sup>B</sup>                 |
| Hepatitis A<sup>B</sup>         | Cyclospora cayatanensis<sup>B</sup>               |
| Yellow fever<sup>C</sup>        | Entamoeba histolytica<sup>B</sup>                 |
| Rabies<sup>C</sup>              |                                                     |

(continued)
Table 11.1 (continued)

| Toxins                                           | Central European tick-borne encephalitis<sup>a</sup> |
|--------------------------------------------------|-------------------------------------------------------|
| Nipah virus<sup>b</sup>                          | Toxoplasma<sup>b</sup>                                |
| Tick-borne encephalitis complex (flavivirus)      | *Giardia lamblia*<sup>b</sup>                         |
| *Coccidioides posadasi*b                          |                                                       |
| *Coccidioides immitis*a                           |                                                       |
| Microsporidia<sup>b</sup>                         |                                                       |

Listing of the biological agents considered to be a threat to human health as a compilation from a number of sources including the (1) select agents and toxins provided by the U.S. Department of Health and Human Services (DHHS), Centers for Disease Control and Prevention (CDC) and the U.S. Department of Agriculture (USDA) and (2) the priority pathogens from the National Institutes of Health/National Institute of Allergy and Infectious Diseases (NIAID) (www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research/CatA.htm).

<sup>a</sup>Select Agents and Toxins designated by DHHS/CDC  
<sup>b</sup>Overlap select agents and toxins that are designated and regulated by both DHHS/CDC and the U.S. Department of Agriculture (USDA)  
<sup>c</sup>Select agents designated and regulated by the USDA  
<sup>A, B, C</sup>NIH/NIAID priority pathogens group A, B or C

11.2.3 Category C Agents

Presently, Category C biological agents are not considered to be high risk, but rather as possible emergent diseases that could pose a threat to public health. The Category C comprises the Nipah virus and hantavirus, yellow fever, influenza, rabies, tick-borne encephalitis viruses, severe acute respiratory syndrome-associated coronavirus (SARS-CoV), as well as certain other types of drug/antibiotic-resistant pathogens, such as tuberculosis-causing mycobacteria.

In addition, the NIAID Category C agents include pathogens commonly found circulating in the general population as Hepatitis A and C and HIV.

11.3 Immunology: Origins and Development

Immunology is a rather young science (a little more than 60 years old, with this explicit name). However, the first information on the existence and validity of immune defenses go back indeed to Edward Jenner with his “vaccination” practices in the 1790s and probably to some older but analogous Chinese medicine practices [14].

The first “immunological” experiment by Jenner is something that would not be ethically feasible today by any medical deontological rules! He started from the observation that English milkmaids who caught cowpox (a benign form of skin eruptions from contacts with diseased cows) did not develop the human form of the smallpox disease; therefore, he voluntarily and deliberately exposed his gardener’s son (James Phipps) to biological materials from cowpox pustules (causing a fever illness in the recipient); after recovery, Jenner challenged the boy with human smallpox, verifying his attained “immunity” also to the human disease [15].
Jenner is therefore considered the father of experimental “immunology.” No mechanistic explanations of that experiment were, however, possible at that time: only about 100–150 years later we started discovering antibodies and immune system functions allowing us to understand what was biologically happening at that time in the milkmaids (and at any other time in “immune” individuals) and in the “vaccinated” individuals like Jenner’s gardener’s son.

All the developing “immunological” science, enriched later through microbiology, biochemistry, physiology and pathology studies in the following decades, recorded more and more details in the functioning of the defense mechanisms of mammals and humans, having the most complex and efficient forms of immunological defense against foreign agents entering the body.

The immune system is in fact like an “eye within” the body, controlling that nothing extraneous is biologically active in each individual organism, and therefore, recognizing effectively “self” from “not-self”, so that replication of self-cells are not contaminated by “foreign” biological agents and that any extraneous biological entity will be eliminated by soluble “light” weapons (antibodies) and by “heavy” killers (immune cells and macrophages, interacting and collaborating in the task).

The key steps of an immune reaction are substantially three: (a) internalization of foreign particles by macrophages (MPH) degrading and “presenting” them to T-helper lymphocytes (T_h-cells) which in turn start an elaborated attack, mostly through either (b) synthesis of soluble “light” weapons (antibodies, humoral response by B-cells) or (c) development of cellular “heavy” weapons (killer T-lymphocytes T-K cells, with cellular or cytotoxic response); in many cases, we observe the activation of both responses (b + c), depending on the needs. The key steps of an immune reaction are illustrated in the following Fig. 11.1.

Recently, it has been shown that activated T-cells tend to aggregate, like a swarm of bees, exchanging information, useful to coordinate the immune response (i.e., to elaborate coordinated defense plans): this is the visual demonstration of the complex molecular dialogue taking place within the various families of immune cells collaborating to the full enactment of defense mechanisms.

When invaders are present in body fluids or the extracellular domains, like most bacteria, they can be attacked by antibodies, through specific surface recognition. However, when the invaders are instead of the intracellular type, like viruses and some bacteria (like TBC), they must instead be attacked by special killer lymphocytes (cytotoxic TK-cells), which destroy all the self-cells harboring the intruders, together with their content. Nevertheless, viruses anyway can also transit the body fluids; consequently, also antibodies are produced against them.

In most responses, both humoral (antibodies) and cellular attacks are deployed. The immune response, moreover, is specific and potentiated by memory: therefore, a second (or further) encounter with the same foreign agent (antigen) produces a stronger and quicker defense reaction. Details of the immune mechanisms are continuously discovered. So, this discipline is still far from being completely described and understood. New details are continuously added and better focused by immunology researchers in time [16].
11.4 Assessing Degrees of Mucosal Involvement

In order to develop a vaccine for biodefense, it is primordial to know the difference between biological agents that elicit mucosal infections, and the biological agents that simply exploit mucosal tissues as a means to gain access to the systemic compartment. Consequently, mucosal immunity likely plays an essential role in preventing and clearing infections. This is why vaccines against these agents have to involve mucosa-associated lymphoid tissues.

On the other hand, situations where the mucosa functions act solely as the port of entry, systemic immunity is likely to be sufficient to control infection. For example, we can consider the case of anthrax. Although the *B. anthracis* spores are highly infectious by aerosol, the vegetative bacteria generally do not multiply locally. Somewhat, after inhalation of the spores, the bacteria circulate systemically via the lymphatics and the circulatory system. Then, within the systemic compartment, *B. anthracis* germinates and produces two toxins, which account for the lethality associated with this infection [17]. For these reasons, protective immunity to *B. anthracis* is associated primarily with anti-toxin serum IgG antibodies. The mucosal defense is of little (if any) importance in controlling anthrax.

Contrary to protective immunity, mucosal immunity plays an important role in controlling infections caused by two other Category A bacterial pathogens, notably *Y. pestis* and *F. tularensis* that are the main cause of mucosal and systemic complications following inhalation [18].
For many of the other Category A–C agents, it should be noted that the route of infection transmission cannot occur via the respiratory tract. Thus, the actual participation of the mucosa in the pathogenesis of the infection process is not usually known. Therefore, the initial host interaction and the subsequent pathophysiology will follow known established clinical outcomes. In addition, there could be a lack of clinical data that defines the aerosol-related disease adequately. An example of one such agent is Staphylococcal enterotoxin B (SEB), which is a member of the superantigen family of toxin. Hence, SEB forms “bridges” between the Major Histocompatibility Class II molecules on antigen-presenting cells and T cell receptors on specific subsets of CD4⁺ and CD8⁺ T cells. And as a result of this SEB binding, the T cells start to release massive amounts of proinflammatory cytokines and undergo hyper-proliferation, which ultimately results in their depletion [19, 20].

For the alphaviruses, the infection and associated pathogenesis depend on the route of exposure. It is assumed that exposure to aerosols induce disease directly via the olfactory bulb, whereas experimental infection via ingestion causes a disseminated viremia prior to nervous system engagement and encephalitis.

11.5 Prevention to Favor Defense and Immunity Success

To control infections and favor human immune responses, it is essential to adhere to the following defenses:

1. to reduce microorganism proliferation (through the use of bacteriostatic drugs) or kill the germs (disinfection, use of bactericidal medications);
2. to control the vectors diffusing the infection (insects, arthropods, birds, rats, etc.);
3. to immunize the potential hosts (vaccinations) preventively: this requires public health planning, technology, costs and time;
4. to administer preformed antibodies (serotherapy): this also requires technology, costs, and time.

11.5.1 Actions Against Germs

Concerning prevention defense #1, the use of chemotherapeutic antibiotics, which have a multitude of modes of action mechanisms, is recommended. However, as germs tend to develop resistance to them, it is evident that fewer effective antibiotics are available today. It should be prevention defenses #2–4 require stringent and effective world operations against biological vectors, which could be transported by insecticides, biological competitors – fungi, bats, genetics can also be beneficial [21, 22]. But the best possible actions to combat biothreats are the use of vaccines (if available and if time allows) and of immune sera (if available).
A vaccine is always preferable because it induces an active response and an advantageous state of immune memory in the individual, with minimal side effects (active immunization). A serum specific for a given antigen can be life-saving (serotherapy, immediately active), but has some side effects (since it introduces heterologous proteins—for instance, horse immunoglobulins, which in turn will be immunologically eliminated) and does not have lasting protection (passive immunization).

Modern life has created some extra occasions for germs and some “new” diseases [22]: air conditioning apparatuses for instance (if not properly cared and cleaned) are a new ideal environment for bacteria; because of dirt and humidity they can foster the growth of airborne bacteria never seen before as human pathogens: *Legionella pneumophyla*. The story of Legionaries disease (a fatal lung infection by *Legionella*) is very instructive for microbiologists and epidemiologists. Human behaviors (homosexual intercourses, exchange of syringes among intravenous drug users, and frequency of air travel) have extended the contagion of formerly rare infections like HIV. Also, in this case, the facts are dramatically instructive for medicine and epidemiology.

Transfusions of unscreened blood have also diffused hepatitis and HIV viruses. Centralized processing of foods has sometimes diffused a contagion of food-borne infections (*E. coli*) [21]. Airplanes have replaced ships for human travel and are at the center of attention for human communicable diseases spreading (Influenza, SARS, etc.). Many more people are traveling today to remote and tropical areas than before (forests, wilderness). This can expose more populations (even at home, on the return) to rare insects and microbes (see cases of malaria, Ebola virus, Marburg virus, etc.). In addition, economic development expands contacts: mining, forestry, agriculture in new tropical areas with recent deforestation.

Additionally, the increasing number of subjects with immunodeficiency diseases or immunosuppressant therapies (for transplants) strengthen the probability of new communicable agents to infect people, survive and propagate in modern societies (opportunistic infections, with possible mutations in progress.

This entire prospect of new possible pandemics constantly increases especially with the emerging new variety of infections such as new bacterial and viral pathogens as the Rotavirus, *Cryptosporidium parvum*, *Legionella pneumophyla*, *Ebola* virus, *Hantaan* virus, *Campylobacter jejuni*, HTLV virus, HIV, *Helicobacter pylori*, Herpesvirus-6 and -8, virus *Guanarito*, virus *Sabia*, nCoV-MERS virus, COVID-19, to name just some of the most relevant and recent [22].

### 11.6 Preparation of Vaccines

What is a vaccine? In a dangerous disease caused by microorganisms and “foreign” not-self substances (an “antigen”), it may be possible to raise a vaccine, that is an innocuous preparation of that “antigen” which can induce the production of an immune response and create immune memory in the subjects we want to protect.
A vaccine can be developed in many ways: using killed (denatured) or non-replicating pathogens (viruses), recombinant protein antigens, live, attenuated (less harmful) strains of pathogens (for instance cowpox virus to protect against smallpox).

Vaccines and their uses have shown to be very effective in eradicating smallpox. Indeed, the extinction of the smallpox disease on earth is one of the best success of global vaccine campaigns. Similarly, we can say the same thing concerning polio. Hence, the near extinction of disease in the world, except in some nomad populations and critical areas of today’s world, like Syria and also, surprisingly, of remote China provinces [22].

### 11.6.1 Requirements for Vaccine Preparation

Requirements for vaccine preparation are the following:

(a) An innocuous preparation of the antigen (+/− adjuvants);
(b) a suitable biologic model in which to test the effectiveness of the immunization procedure;
(c) a reduced series of administrations able to induce a long-lasting response (number of boosters required): polio (very long-lasting), as opposed to tetanus (lasts only some years, many boosters required);
(d) an easy and affordable procedure for preparing quickly a large amount of product to use in case of necessity [22].

### 11.6.2 Different Method of Vaccinations

The most used method of vaccination generally can be divided as follows:

1. Using killed whole germ preparations (influenza; polio type Salk; Hepatitis A; rabies; pertussis; cholera);
2. Using live attenuated germ preparations (tuberculosis; mumps-parotitis-rubella; polio type Sabin; yellow fever; variola; typhus and lately also against Ebola and Marburg viruses);
3. Using purified antigens component of the infective diseases (meningitis –using antigenic subunits of *Haemophilus influenzae b*; acellular pertussis vaccine; tetanus-diphtheria anatoxins);
4. Using recombinant DNA vaccines (surface antigen of hepatitis B);
5. Using naked DNA vaccines (against many different germs, not yet widely used); and
6. Finally, using vaccines of genetically modified plants (against many different germs, not yet widely used) [22].
The technology of vaccine preparations is continuously evolving, in order to improve our capacity to prepare quickly enormous amounts of vaccinating doses from microorganisms soon after their isolation in newly appearing forms or strains. For viruses, for instance, an important step forward has been the technology of reproducing the organisms on cell cultures rather than on fertilized chicken eggs.

11.6.3 Vaccination Campaigns: How Best Use Vaccines

Mass vaccination campaigns have taken place several times in history, like those of 1947 against smallpox, in which the citizens of New York City stood in long lines to be vaccinated at a rate of eight persons per minute, and like those of several countries against polio (after the Sabin vaccine introduction, in 1960), with periodic vaccination days organized to reach many secluded groups of citizens living in remote areas [22].

The realities, however, are that the existence of a vaccine is not enough to prevent a worldwide pandemic; more testing is needed before a new vaccine may be offered to the public; for vaccines prepared on chicken eggs, the successful mass production depends on the availability of the eggs, indeed; obstacles include an organizational system for distribution and timely supply of doses, where and when they are needed.

Good news for scientists and biologists is that since the September 2001 terrorist attacks in the USA (anthrax spores in the mailings), the employment needs of biologists have increased remarkably (production of vaccines, enactment of protection plans, treatment schemes for infections, etc.) [21, 22].

The field of immunology, in particular for vaccine research and development, continues to be of high impact in modern medicine and high relevance in the contrast of bioterrorism.

To conclude, science is here to help; to be of use; to spread not infections but knowledge, across different cultures and nationalities. Our role of scientists is mostly that of being “pontifices” (Latin “pontes facientes,” which means “bridge makers” across cultures and nationalities). For this reason, it has been a real pleasure to exchange our knowledge here and to discuss it together, among so many different people and so broad scientific expertise.

11.7 Development of Biothreat Vaccines

The development of vaccines and other countermeasures against the diverse biological agents that can be considered posing potential biothreats to public health is a daunting challenge for the scientific community. Licensed vaccines against both anthrax and smallpox that protect against aerosol transmission are available. An existing licensed plague vaccine is protective against flea-transmitted disease but not against aerosol challenge in animal experiments or pneumonic plague. This vaccine is in limited supply, and the manufacturer has recently ceased production.
11.7.1  Glycoconjugate Vaccines Used for Prevention from Biological Agents

As mentioned before in 1796, Edward Jenner discovered that the inoculation with cowpox was able to protect against smallpox infection. Since then, different vaccines were developed to prevent infectious diseases [23, 24]. It was found that the protection conferred by these vaccines was due to adaptive immunity (cellular and/or humoral immunity) [23–26]. Unlike adaptive immunity, innate immunity does not recognize every possible antigen. Instead, it is designed to recognize the microbial molecules that are essential for the survival of the pathogens. These unique microbial molecules are called pathogen-associated molecular patterns (PAMPS) [25, 26].

PAMPS include lipopolysaccharides (LPS, also called endotoxin) from the gram-negative cell wall, peptidoglycan lipoteichoic acid from the gram-positive cell wall, flagellin of bacterial flagella, the sugar mannose (a terminal sugar common in bacterial, viral or fungal glycolipid and glycoprotein), bacterial or viral unmethylated CpG DNA, double-stranded and single-stranded RNA from viruses and glucans from fungal cell wall [25, 26].

Several pathogens and tumor cells exhibit unique glycan structures on their cellular membrane surfaces. For example, bacteria, microbes, and viruses all possess a cell wall consisting of a plasma membrane and a capsule which were formed of glycoproteins or complex polysaccharides [27].

These carbohydrate antigens can be used as targets for the development of carbohydrate vaccines. In the case of pathogenic bacteria, one has to distinguish Gram-positive to Gram-negative bacteria that possess respectively, either capsular polysaccharides (CPS) and/or lipopolysaccharides (LPS) that are implicated in all virulence factors [28, 29]. The LPS are located on the outer membranes of Gram-negative bacterial cells. The study of the LPS revealed that it is composed of an amphiphilic macromolecule, corresponding to an external core oligosaccharide (O-specific chain, or hydrophilic antigen) and an internal core oligosaccharide covalently linked to the lipid A [30]. Knowing that the oligosaccharide portion confers the immunological properties to the bacteria, different portions of the LPS have been tested for vaccine development [31].

11.7.2  Lipopolysaccharide (LPS)

The lipopolysaccharides are located on the outer membranes of Gram-negative bacterial cells. Several studies on the isolation and structure and composition determination of LPS were reported [30, 32]. It was observed that LPS are composed an external polysaccharide consisting of repeating of identical sugar oligosaccharide units, called the O-specific chain, and an internal core oligosaccharide covalently attached to a lipid A. The lipid A is a glycolipid composed by a β-D-(1 → 6) GlcN
disaccharide in which O-3, O-3′, O-4′, C 2 -N and C 2 -N′ are acylated with different C:12 and C:14 fatty acids. The lipid A confers the toxicity to the LPS. In addition, it has to be noted that the O-specific oligosaccharide that corresponds to a sequence of oligosaccharide units is unique for each bacterial serotype and provides an immunological property to the bacteria. As a result, several studies involving different moieties of lipopolysaccharides were carried out for the development of vaccines and drugs [31, 33, 34].

11.7.3 LPS-Derived Vaccines or LPS-Protein Neoglycoconjugates

Various studies were carried out on the use of LPS derivatives as vaccines. It was found that the immune reaction relies mainly on the LPS, and it has been efficiently utilized for vaccination. However, the use of the LPS alone for vaccination is not efficient, as, because of its small size, it is not recognized by the immune system as it has resistance to non-specific host immunity such as complement system and resistance to specific host immunity (poor antibody response) [35, 36].

However, when conjugated to a protein carrier, the LPS is able to induce an immunological reaction and an extended immunity. Landsteiner’s group was the first to utilize carbohydrate-protein conjugates as immunogens [37, 38], and lately, it was discovered that they could induce a strong antibody reaction [39]. Landsteiner’s group was also first to refer to the carbohydrate-protein conjugate as a hapten [37, 38].

Figure 11.2 is representing the immune response generated by oligosaccharide antigen-carrier protein vaccines [40]. The interaction of the glycoconjugate vaccine (Fig. 11.2) with the B cell receptor (BCR) stimulates the lymphocytes and results in the activation of plasma B cells to secrete immunoglobulins, while the neoglycoconjugate vaccine is recognized by the polysaccharide specific B cell receptors. The carrier protein will enter the B cell by phagocytosis. Then, the degradation of the protein carrier in B cells will be done by lysosomal enzymes into short peptides called epitopes. At that time these epitopes will be recruited by a special protein called major histocompatibility protein II (MHC II) that are presented at the cell surface of antigen-presenting cell (APC) or B cell and interact with the carrier-peptide-specific T cells (helper T cell) which send a signal to produce polysaccharide specific plasma cells and polysaccharide specific memory B cells [40].

The synthesis of efficient glycoconjugate vaccines has been challenging, since their efficacy relies on different factors, such as the saccharide size, the average number of saccharide chains per conjugate molecule, the nature of the carrier and the distance between the saccharide and the protein in the formed glycoconjugate (Fig. 11.3) [41–44].
Different methods have been used for the synthesis of carbohydrate antigen-protein neoglycoconjugates. One of these methods consisted of the use of the squaric acid chemistry for the single-point attachment of carbohydrates to proteins [45–48]. Tietze et al. used squaric acid diethyl esters for the conjugation [46], while it has also been reported that squaric acid dimethyl esters [49, 50], as well as didecyl squarate [51], were used for the single-point attachment of carbohydrates to proteins. In addition, Kamath et al. used squaric acid amide ethyl esters for the conjugation of oligosaccharides to protein. They monitored the conjugation using matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) [52].

More recently, the group of Kováč also utilized the squaric acid chemistry to conjugate different carbohydrate antigens to a protein carrier [53–55]. They used this strategy to conjugate the synthetic tetrasaccharide side chain of the *Bacillus anthracis* exosporium to the bovine serum albumin (BSA) protein [54].

![Fig. 11.2 Generation of the immune responses against oligosaccharide antigen-carrier protein conjugate vaccines [40]](image)

![Fig. 11.3 Composition of the neoglycoconjugate vaccine](image)
11.7.4 Molecular Weight and Carbohydrate-to-Protein Ratio Determination

The aim of determining the molecular weight and the carbohydrate-to-protein ratio of a carbohydrate-protein neoglycoconjugate is to define the number of carbohydrates that are incorporated in the protein carrier, as a result of the conjugation. Two main methods are currently used for the molecular weight determination carbohydrate-protein glycoconjugates: matrix-assisted-laser-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) [56, 57] and surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) [53–55, 58–60]. Both of these methods allow determining the carbohydrate-to-protein ratio of the neoglycoconjugates by comparing the molecular weight of the protein before and after the conjugation to that of the neoglycoconjugate.

11.7.5 Mass Spectrometry Methods for the Characterization of Carbohydrate Vaccines

Mass spectrometry has emerged as a powerful technique for the characterization of different biomolecules ranging from small molecules to larger molecules. Thus, mass spectrometry is extensively used in proteomics [61], glycomics [62], metabolomics [63], lipidomics [64], and in oligonucleotides [65] analysis.

Initially, the exploring of MALDI-TOF-MS of the different hapten-BSA glycoconjugate vaccines allowed us to determine the hapten-to-BSA ratios. Then the glycoconjugate vaccine samples were then digested and analyzed by MALDI-TOF/TOF-MS/MS and LC-ESI-QqTOF-MS/MS for the determination of glycation sites.

The digestion was done by two different enzymes; trypsin, which will not be able to digest or react with the glycated lysines of the protein, and the other enzyme was GluC V8 endoproteinase which is known to digest proteins at C-terminus of the aspartic acid and glutamic acid residues. Finally, the MS/MS spectra will be submitted to the MASCOT library to get the matched and non-matched peptides.

11.7.6 MALDI-TOF-MS

Matrix-assisted laser desorption ionization mass spectrometry has been successfully used for the determination of the molecular weight of biomolecules, such as proteins, oligosaccharides, and glycoproteins [56–58]. Kamath et al. used MALDI-TOF-MS to characterize neoglycoconjugates formed by the conjugation of oligosaccharide amines to carrier proteins by the aim of diethyl squarate [59]. The MALDI-MS was recorded in linear mode and with positive ion detection. Figure 11.4 displays the MALDI-TOF-MS analysis of BSA (A) and the following
oligosaccharide-BSA glycoconjugates: (B) GlcNAc-BSA (carbohydrate-BSA ratio (n) = 8.4); (C) lactose-BSA (n = 3.5); (D) lactose-BSA (n = 8.2); (E) Fucal-2Galfl-3[Fucal-4]GlcNAc-BSA (n = 11); (F) Fucal-2Fucal-3GalNAc-BSA (n = 13). The MALDI-TOF-MS spectrum of BSA was used as a calibration standard. Then the analysis allowed to reveal the average carbohydrate-BSA ratios for the hapten-BSA neoglycoconjugates (Fig. 11.4).

Fig. 11.4  MALDI-TOF spectra of: (a) BSA calibration standard; (b) GlcNAc-BSA (n = 8.4); (c) lactose-BSA (n = 3.5); (d) lactose-BSA (n = 8.2); (e) Fucal-2Galfl-3[Fucal-4]GlcNAc-BSA (n = 11); (f) Fucal-2Fucal-3GalNAc-BSA (n = 13) [58]
11.7.7 SELDI-TOF-MS

Surface-enhanced laser desorption ionization time-of-flight mass spectrometry (SELDI-TOF-MS) has been extensively used for biomarkers discovery [59], and cancer diagnosis [60]. This technique consists in using a modified target for the immunoaffinity purification of proteins before analysis.

The group of Kováč successfully used this analytical method to monitor the conjugation of synthetic carbohydrate antigens to different protein carriers [53–55, 66]. Figure 11.5 shows the SELDI-TOF-MS analysis with a ProteinChip® System of a neoglycoconjugate prepared by the dialkyl squarate chemistry attachment of the hexasaccharide of *Vibrio cholerae* O:1 to the BSA [65]. This technology allowed the analysis of neoglycoconjugates at different reaction times by taking an aliquot of the reaction mixture, in the picomolar concentration range, and analyzing it without any purification step. In addition, this method was found to be fast, and only a small amount of sample is used for analysis. The same group followed the progress of conjugation of the hexasaccharide (exact mass: 1780.79 Da) to the BSA protein (molecular mass: 66,430 Da) at different reaction times [65]. It was observed that the conjugation rate increased with the reaction time. In addition, the SELDI-TOF-MS also allowed observing that after a reaction time of 7 h, the fine structure the peak of the neoglycoconjugate (Fig. 11.6) shows the polydispersity of the neoglycoconjugate formed from the hexasaccharide and BSA.

Moreover, after the conjugation is complete, the excess oligosaccharide can be recovered for further use, allowing an economy of labor and time for the preparation of synthetic oligosaccharides and their conjugation [66].

11.7.8 Glycation Sites Determination

The glycation site’s determination of carbohydrates-protein neoglycoconjugates is usually carried out by first digesting the neoglycoconjugate using a protease, such as trypsin or GluC V8 endoproteinase, followed by MALDI-TOF-MS/MS or liquid chromatography-tandem mass spectrometry [67–72].

It has to be noted that during the tandem mass spectrometry analysis of the glycoconjugate digests, the identification of the glycated peptides is confirmed by the presence of diagnostic product ions of the carbohydrate in the mass spectrum.

In addition, the tandem mass spectrometry analysis also reveals the sequence of the peptide through diagnostic product ions of the peptide moiety of the glycated peptide. The combined information allows the unambiguous characterization of the carbohydrate-peptide and the glycation site identification. It has to be noted that during the tandem mass spectrometry analyses of the glycated peptides, the product ions corresponding to the fragmentation of the peptide portion were identified using the nomenclature established by Roepstorff et al. and lately modified by Johnson and coworkers [73, 74], and the product ions resulting from the fragmentation of the
carbohydrate moiety was assigned using the nomenclature introduced by Domon and Costello, as A, B, C, X, Y, and Z [75]. An example of the mass spectrometry characterization of neoglycoconjugate vaccines is discussed in the next section.

Fig. 11.5 The progress of conjugation of the hexasaccharide of Vibrio cholerae serotype Ogawa (exact mass, 1780.79 Da) and BSA (molecular mass 66,430 Da) as revealed by monitoring the reaction by SELDI-TOF MS. Spectrum A was taken at the onset of the reaction (t-0); spectra B-G were taken at 1, 3, 7, 9, 27, and 54 h, respectively [66]
**A Typical Example of the Mass Spectrometry Characterization of an Anthrax Biothreat**

*Bacillus anthracis* is a Gram-positive bacterium that causes anthrax to both humans and animals [76]. The formation of endospores [77] at the maturity stage of the bacterium allows protection against severe conditions, such as extreme temperatures, radiations, physical damages, and chemicals [78]. *Bacillus anthracis* is the etiologic agent of anthrax that can be used as a biological weapon [79, 80]. Indeed, *Bacillus anthracis* a pathogen that is lethal in most cases for both humans and animals. There are different *Bacillus anthracis* strains, among those, 89 of them were identified, such as the Sterne strain [81], the Vollum strain [82], the Ames strain [80, 83], and the H9401 strain [84]. The last one has also been studied for the development of anthrax vaccine [84]. In addition, *Bacillus anthracis* has been extensively studied to understand its pathogenesis, identifying new biomarkers and vaccines design [85]. The capsular polypeptide (polyglutamic acid) of the *Bacillus anthracis* has been targeted for the development of synthetic vaccines [86].

Daubenspeck et al. reported the structure of the tetrasaccharide side chain of the collagen-like region of the major glycoprotein of the *B. anthracis* exosporium [87]. Their findings were that the upstream terminal of the tetrasaccharide corresponds to the sugar anthrose [4,6-dideoxy-4-(3-hydroxy-3-methylbutyramido)-2-O-methyl-D-glucopyranose].

The group of Kováč prepared a vaccine composed of a synthesized tetrasaccharide side chain of the collagen-like region of the major glycoprotein of the *B. anthracis* exosporium (MW = 950.43 Da) attached to the BSA using the squaric acid chemistry [54]. The conjugation led to the formation of vaccines with different carbohydrate: BSA ratios.

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**Fig. 11.6** The fine structure of the peak D (Fig. 11.5) showing the polydispersity of the neoglycoconjugate formed from the hexasaccharide 4 and BSA after 7 h of reaction time. For further details, see text [66]
11.8.1 Carbohydrate: BSA Ratio

MALDI-TOF-MS analysis of one of these synthetic vaccines allowed to observe the following protonated molecular ions: \([M + H]^+\) at \(m/z\) 71,448.36 and \([M + 2H]^{2+}\) at \(m/z\) 35,730.08. The molecular weight of this vaccine was thus found to be 71,447.36 Da. As the molecular weight of the synthetic tetrasaccharide side chain of the collagen-like region being 950.43 Da, the carbohydrate: protein ratio of the synthetic vaccine was determined to be 5.4:1.

11.8.2 Glycation Sites Determination

The determination of the glycation sites of the hapten-BSA vaccine neoglycoconjugate was carried out by enzymatic digestion with the trypsin and/or GluC V8 proteases, followed by the MALDI-MS/MS and LC-MS/MS analysis of the digests.

The enzymatic digestion of the hapten-BSA glycoconjugate and MALDI-MS/MS, as well as LC-MS/MS analyses, were carried out as previously described [67–71].

11.8.2.1 MALDI-TOF/TOF-MS/MS

The spectra obtained during the MALDI-TOF/TOF-MS/MS analysis of the tryptic and GluC V8 digests (Fig. 11.7) were submitted to the MASCOT library to identify by PMF the peptides matching to the BSA. Two Serum Albumin protein isoforms from the *Bos taurus* species were identified for the tryptic and GluC V8 digests: the serum albumin precursor (gi|1,351,907) and the serum albumin (gi|74,267,962). The detected peptides that were not identified in the database were analyzed by tandem mass spectrometry. The MALDI-MS/MS analysis of the tryptic digests allowed to identify three glycated peptides corresponding to BSA peptides with an increment of 950 Da to their original molecular mass, namely: ALK*AWSVAR at \(m/z\) 1951.0130 (Fig. 11.8), VT*K*CCTESLVNR at \(m/z\) 2416.1372, and QNCDQFEK*LGEGYGFQNALIVR at \(m/z\) 3478.6429 (glycation represented with an asterisk on the lysine residue).

The high-energy CID-MS/MS analysis of the glycopeptide ALK*AWSVAR (Fig. 11.8) at \(m/z\) 1951.0130 afforded a series of product ions corresponding to the entire precursor ion that losses different carbohydrate portions, the monosaccharide \(B_1\) (−259 Da), disaccharide \(B_2\) (−405 Da), trisaccharide \(B_3\) (−551 Da) and/or the tetrasaccharide \(B_4\) (−697 Da), leading respectively to the formation of the following productions: \(Y_3^+\) at \(m/z\) 1691.8829, \(Y_2^+\) at \(m/z\) 1545.8376, \(Y_1^+\) at \(m/z\) 1399.7768 and \(Y_0^+\) at \(m/z\) 1253.7010 (Fig. 11.8). The mass difference between \(Y_3^+\) and \(Y_3^+, Y_2^+\) and \(Y_1^+, Y_1^+\) and \(Y_0^+\) were found to correspond to one \(\alpha\)-L-rhamnopyranosyl unit (146 Da). Moreover, product ions resulting from the fragmentation of the same
Fig. 11.7 MALDI-MS analysis of the glycoconjugate trypsin digests (a) and GluC V8 endoproteinase digests (b) [69]
tetrasaccharide were observed: C₁₇H₂₆N₃O₄⁺ at m/z 336.1242, B₁⁺ at m/z 260.0739, [B₁ − H₂O]⁺ at m/z 242.0709, ²₅A₁⁺ at m/z 230.0719, C₁₁H₁₆N₃O₂⁺ at m/z 222.0670 and [C₁ − C₅H₁₁NO₂]⁺ at m/z 159.0486 (Fig. 11.8). Accordingly, the fragmentation of the carbohydrate portion allowed to confirm its structure and contributed to

![Diagram](image_url)

**Fig. 11.8** (a) MALDI-TOF/TOF-MS/MS spectra of the glycated peptide ALK*AWSVAR (Lys 265) at m/z 1951.0130. (b) Different product ions involving the fragmentation of the carbohydrate hapten observed during the MALDI-TOF/TOF-MS/MS analysis of the glycated peptide ALK*AWSVAR (Lys 235) at m/z 1951.0130 [69]
establishing a diagnostic fragmentation signature of the synthetic tetrasaccharide. In addition, the fragmentation of the peptide portion led to the formation of b- and y-product ions that allowed the determination of the sequence of the glycated peptide.

However, some peptide product ions had the particularity to have lost the carbohydrate portion but were still attached to the spacer-squaric acid chain: \([b_3 - B_4]^+ \text{ at } m/z 565.2661, [b_4 - B_3]^+ \text{ at } m/z 636.2715 \text{ and } [y_7-B_2]^+ \text{ at } m/z 1361.7265\]. Thus, the identified glycated peptides ALK*AWSVAR at \(m/z\ 1951.0130\), VTK*CCTESLVNR at \(m/z\ 2416.1372\), and QNCDQFEK*LGEYGFQNALIVR at \(m/z\ 3478.6429\) allowed to determine the glycation site on the following lysine residues: Lys 235, Lys 498 and Lys 420, respectively.

Similarly, the MALDI-MS/MS analysis of the GluC V8 digests afforded different glycated peptides: LCK*V ASLRE at \(m/z\ 2025.0186\), YAVSVLLRLAK*E at \(m/z\ 2311.2476\) and YAVSVLLRLAK*EYE at \(m/z\ 2603.3484\), allowing to identify the following glycation sites on lysine residues: Lys 100 and Lys 374. To sum it up, only five glycation sites were identified during the MALDI-MS and MS/MS analyses of the tryptic and GluC V8 digests of the hapten-BSA glycoconjugate: Lys 100, Lys 235, Lys 374, Lys 420 and Lys 498.

11.8.2.2 LC-MS/MS

The second approach for the determination of the glycation sites of the vaccine neoglycoconjugate was the LC-MS/MS analysis of the tryptic and GluC V8 digests. It was noted that the LC-MS/MS analysis of peptides has the advantage of minimizing the ionization suppression effect comparing to the MALDI-MS/MS analysis [88, 89].

The data of the LC-MS/MS analysis of the tryptic and GluC V8 digests were submitted to the MASCOT library and matched two serum albumin protein isoforms: the serum albumin precursor (gi|1,351,907) and serum albumin (gi|74,267,962) from \(Bos taurus\).

For the tryptic digests, the BSA sequence coverage was found to be 57% for the serum albumin precursor from \(Bos taurus\) (gi|1,351,907) and 58% for serum albumin protein from \(Bos taurus\) (gi|74,267,962). The LC-MS/MS analysis of the hapten-BSA tryptic digests allowed the identification of 18 glycated peptides, reported in Table 11.2.

The low-energy CID-MS/MS analysis of the extracted precursor ions of the glycated peptides allowed to localize the glycation sites on the following 18 lysine residues: Lys 140, Lys 155, Lys 156, Lys 204, Lys 211, Lys 228, Lys 235, Lys 304, Lys 374, Lys 401, Lys 420, Lys 437, Lys 455, Lys 463, Lys 495, Lys 498, Lys 547 and Lys 559.

The LC-MS/MS analysis of the hapten-BSA vaccine GluC V8 digest allowed the identification of the serum albumin from \(Bos taurus\) (gi|74,267,962) with a sequence coverage of 42% and the precursor serum albumin from \(Bos taurus\) (gi|1,351,907) with a sequence coverage of 45%, in the MASCOT database. Table 11.3 displays the identified glycated peptides during the LC-MS/MS analysis of the GluC V8
Table 11.2 Tryptic glycopeptides identified of the bovine serum albumin protein by LC-ESI-QqTOF-MS/MS analysis of the hapten-BSA glycoconjugate [69]

| Precursor ion m/z (Charge) | Mr (expt) | Mr (calc) | Deviation | Missed Cleavage | Peptide |
|---------------------------|-----------|-----------|-----------|------------------|---------|
| 729.8826 (+2)             | 1457.7506 | 1457.7389 | 0.0117    | 1 KHK’P (Lys 559) |
| 733.3170 (+2)             | 1464.6194 | 1464.7698 | −0.1504   | 1 QIK’K (Lys 547) |
| 770.3757 (+2)             | 1538.7369 | 1538.7338 | 0.0031    | 1 ADEK’K (Lys 155) |
| 807.9138 (+2)             | 1613.8129 | 1613.7964 | 0.0165    | 0 K’FWGK (Lys 156) |
| 828.0811 (+3)             | 2481.2213 | 2481.2005 | 0.0208    | 2 LKECCDK’PLEK (Lys 304) |
| 832.7770 (+3)             | 2495.3092 | 2495.3146 | −0.0054   | 1 LK’HLVDEPQNKLI (Lys 401) |
| 863.7965 (+3)             | 2588.3677 | 2588.3572 | 0.0105    | 0 K’VPQVSTPTLVEVSR (Lys 437) |
| 883.9655 (+2)             | 1765.9164 | 1765.9085 | 0.0079    | 1 SLGK’VGTR (Lys 455) |
| 922.0941 (+3)             | 2763.2605 | 2763.2493 | 0.0112    | 1 LAK’EYEATLLECCK (Lys 374) |
| 969.5086 (+2)             | 1937.0026 | 1936.9868 | 0.0158    | 1 TPVSEK’VTK (Lys 495) |
| 970.4900 (+2)             | 1938.9738 | 1938.9772 | −0.0034   | 1 EK’VLTSSAR (Lys 211) |
| 976.0173 (+2)             | 1950.0201 | 1950.0085 | 0.0116    | 1 ALK’AWSVAR (Lys 235) |
| 990.4710 (+3)             | 2968.3911 | 2968.3886 | 0.0025    | 2 LK’PDNPNTLCDEFKADEK (Lys 140) |
| 1073.0144 (+2)            | 2144.0143 | 2144.0082 | 0.0061    | 1 CASIQK’FGER (Lys 228) |
| 1058.4652 (+2)            | 2114.9159 | 2114.9123 | 0.0036    | 1 CCK’PESER (Lys 463) |
| 1208.5857 (+2)            | 2415.1569 | 2415.1284 | 0.0285    | 1 VTK’CTESLVNR (Lys 498) |
| 1160.2172 (+3)            | 3477.6297 | 3477.6385 | −0.0088   | 1 QNCDQFQK’LGEYGFQNALIVR (Lys 420) |
| 1169.5978 (+2)            | 2337.1810 | 2337.1583 | 0.0227    | 1 GACLLPK’IETMR (Lys 204) |

digests. Thus, the CID-MS/MS analysis of theses glycated peptides allowed to discover 17 glycation sites, localized on the following lysine residues: Lys 65, Lys 75, Lys 88, Lys 100, Lys 117, Lys 151, Lys 183, Lys 197, Lys 256, Lys 266, Lys 304, Lys 309, Lys 336, Lys 374, Lys 420, Lys 455 and Lys 495.
In summary, the LC-MS/MS analysis of both tryptic and GluC V8 digests allowed the identification of a total of 30 glycation sites on the lysine residues (Fig. 11.9a). Mapping these glycation sites on the 3D representation of the BSA (Fig. 11.9b, lysines highlighted in red) permitted to observe that they correspond to lysine residues located at the outer surface of the BSA. In addition, the number of

| Precursor ion | Deviation | Missed | Peptide |
|---------------|-----------|--------|---------|
| m/z (charge)  | Mr (expt) | Mr (calc) | Da | Cleavage | Peptide |
| 698.3598 (+2) | 1394.7051 | 1394.6803 | 0.0248 | 0 | K*LGE (Lys 420) |
| 790.3774 (+2) | 1578.7403 | 1578.7288 | 0.0115 | 1 | K*QEPE (Lys 117) |
| 844.3721 (+2) | 1686.7297 | 1686.7499 | -0.0202 | 1 | EFK*ADE (Lys 151) |
| 894.4637 (+2) | 1786.9128 | 1786.8976 | 0.0152 | 0 | HVK*LVNE (Lys 65) |
| 862.9539 (+2) | 1723.8933 | 1723.8754 | 0.0117 | 1 | VTK*LVTD (Lys 256) |
| 897.4200 (+2) | 1792.8255 | 1792.8176 | 0.0079 | 0 | K*SCHIAE (Lys 309) |
| 902.4801 (+2) | 1802.9456 | 1802.9289 | 0.0167 | 0 | LTKVHK*E (Lys 266) |
| 962.1552 (+2) | 2883.4439 | 2883.4093 | 0.0346 | 0 | VSRSLGKV*GTRCCCTKPE (Lys 455) |
| 987.9658 (+2) | 1973.9171 | 1973.8949 | 0.0222 | 0 | K*VTBCCTE (Lys 495) |
| 989.9697 (+2) | 1977.9248 | 1977.9154 | 0.0094 | 2 | K*QEPERNE (Lys 117) |
| 992.4568 (+2) | 1982.8989 | 1982.884 | 0.0149 | 1 | CCDK*PHEL (Lys 304) |
| 995.4702 (+2) | 1988.9258 | 1988.8912 | 0.0346 | 1 | FAK*TVCADE (Lys 75) |
| 1013.0142 (+2) | 2024.0138 | 2024.0123 | 0.0015 | 0 | LCK*VSLRE (Lys 100) |
| 1048.5225 (+2) | 2095.0305 | 2094.9984 | 0.0321 | 1 | K*SLHTLFGDE (Lys 88) |
| 1097.0595 (+2) | 2192.1045 | 2192.0909 | 0.0136 | 0 | DK*GACLKPKIE (Lys 197) |
| 1124.5018 (+2) | 2246.9890 | 2246.9876 | 0.0014 | 1 | DK*DVCKNYQE (Lys 336) |
| 1336.1446 (+2) | 2670.2746 | 2670.2728 | 0.0018 | 0 | LLYYANK*YNGVFQE (Lys 183) |
| 1156.132 (+2) | 2310.2495 | 2310.2345 | 0.0150 | 0 | YAVSVLLRLAK*E (Lys 374) |

Table 11.3 Glycopeptides identified in the bovine serum albumin protein by LC-ESI-QqTOF-MS/MS analysis of the hapten-BSA glycoconjugate digested with the endoproteinase GluC V8 [69]
the identified glycation sites (30 lysine residues) being higher than the determined (hapten: BSA ratio 5.4:1) of the tetrasaccharide-BSA neoglycoconjugate, it was concluded that the vaccine is composed of a mixture of glycoforms.

11.9 Conclusion

The systematic investigations presented herein constitute a series of versatile examples for the identification of accurate quality control necessary in commercial production of glycoconjugate vaccines against infectious diseases. The glycopeptides isolated and fully characterized during this work may well represent useful reference compounds to be used in standardization analyses.

Moreover, although BSA usually serves a universal model carrier protein for novel conjugation chemistry, we found it perfectly legitimate as a vaccine in mouse
experiments since the monoclonal antibodies isolated from the above conjugates were the basis for fully synthetic carbohydrate-based vaccines [24–27]. This would be particularly true when performed on other more immunogenic protein carriers such as tetanus toxoid and KLH.

References

1. Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Hauer J, McDade J, Osterholm MT, O’Toole T, Parker G, Perl TM, Russell PK, Tonat K (1999) Anthrax as a biological weapon: medical and public health management. Working group on civilian biodefense. JAMA 281(18):1735–1745
2. Inglesby TV, O’Toole T, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Friedlander AM, Gerberding J, Hauer J, Hughes J, McDade J, Osterholm MT, Parker G, Perl TM, Russell PK, Tonat K (2002) Anthrax as a biological weapon, 2002: updated recommendations for management. JAMA 287(17):2236–2252
3. Henderson DA, Inglesby TV, Bartlett JG, Ascher MS, Eitzen E, Jahrling PB, Hauer J, Layton M, McDade J, Osterholm MT, O’Toole T, Parker G, Perl T, Russell PK, Tonat K (1999) Smallpox as a biological weapon: medical and public health management. Working group on civilian biodefense. JAMA 282(22):2127–2137
4. Lovinger S (2002) Addressing the unthinkable: preparing to face smallpox. JAMA 288(20):2530
5. Inglesby TV, Dennis DT, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Koerner JF, Layton M, McDade J, Osterholm MT, O’Toole T, Parker G, Perl TM, Russell PK, Schoch-Spana M, Tonat K (2000) Plague as a biological weapon: medical and public health management. Working group on civilian biodefense. JAMA 283(17):2281–2290
6. Arnon SS, Schechter R, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Hauer J, Layton M, Lillibridge S, Osterholm MT, O’Toole T, Parker G, Perl TM, Russell PK, Swerdlow DL, Tonat K (2001) Botulinum toxin as a biological weapon: medical and public health management. Working group on civilian biodefense. JAMA 282(22):1059–1070
7. Dennis DT, Inglesby TV, Henderson DA, Bartlett JG, Ascher MS, Eitzen E, Fine AD, Friedlander AM, Hauer J, Layton M, Lillibridge SR, McDade JE, Osterholm MT, O’Toole T, Parker G, Perl TM, Russell PK, Tonat K (2001) Tularemia as a biological weapon: medical and public health management. JAMA 285(21):2763–2773
8. Porter RK (ed) (2011) The Merck manual of diagnosis and therapy, 19th edn. Merck Sharp & Dohme Corp., Whitehouse Station
9. Choudhary S, Malik YS, Tomar S (2020) Identification of SARS-CoV-2 cell entry inhibitors by drug repurposing using in Silico structure-based virtual screening approach. ChemRxiv Preprint. https://doi.org/10.26434/chemrxiv.12005988.v1
10. Mantis NJ, Morici LA, Roy CJ (2011) Mucosal Vaccines for Biodefense. In: Kozlowski P (ed) Mucosal vaccines. Current topics in microbiology and immunology, vol 354. Springer, Berlin, Heidelberg
11. Rotz LD, Khan AS, Lillibridge SR et al (2002) Public health assessment of potential biological terrorism agents. Emerg Infect Dis 8:225–230
12. Artenstein AW (2008) New generation smallpox vaccines: a review of preclinical and clinical data. Rev Med Virol 18:217–231
13. Sobel J, Khan AS, Swerdlow DL (2002) Threat of a biological terrorist attack on the US food supply: the CDC perspective. Lancet 359:874–880
14. Bottacchioli F (2002) Il sistema immunitario: la bilancia della vita – Com’Ã¨ fatto, come funziona in salute e in malattia. Tecniche Nuove. ISBN 8848109462, www.tecnichenuove.com
15. Actor J (2014) Introductory immunology. 1st edition - basic concepts for interdisciplinary applications. Academic. ISBN: 9780124200302
16. Abbas A, Lichtman AH, Pillai S (2014) Cellular and molecular immunology, 8th edn. Saunders, ISBN: 9780323316149
17. Leplla SH, Robbins JB, Schneeron R et al (2002) Development of an improved vaccine for anthrax. J Clin Invest 110:141–144
18. Metzger DW, Bakshi CS, Kirimanjeswara G (2007) Mucosal immunopathogenesis of Francisella tularensis. Ann NY Acad Sci 1105:266–283
19. Kappler J, Kotzin B, Herron L et al (1989) V beta-specific stimulation of human T cells by staphylococcal toxins. Science 244:811–813
20. White J, Herman A, Pullen AM et al (1989) The V beta-specific superantigen staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. Cell 56:27–35
21. Murphy K (2011) Janeway’s immunobiology, 8th edition. Garland Science. ISBN-10: 0815342438
22. Abbas AK, Lichtman AH (2010) Basic immunology, 3rd edn. Saunders Kindle Edition ISBN-10: 141605569X
23. Plotkin SA (2008) Vaccines: correlates of vaccine-induced immunity. Clin Infect Dis 47:401–409
24. Heidelberger M, Avery OT (1923) The soluble specific substance of pneumococcus. J Exp Med 38:73–79
25.Ausubel FM (2005) Are innate immune signaling pathways in plants and animals conserved? Nat Immunol 6:973–979
26. Rumbo M, Nempont C, Kraehenbuhl J-P, Sirard J-C (2006) Mucosal interplay among commensal and pathogenic bacteria: lessons from flagellin and toll-like receptor 5. FEBS Lett (12):2976–2984
27. Shetty N, Aarons E, Andrews J (2009) Structure and functions of microbes. In: Shetty N, Tang JW, Andrews J (eds) Infectious disease: pathogenesis, prevention, and case studies. Wiley, London, p 15
28. Corbett D, Hudson T, Roberts IS (2010) Bacterial polysaccharide capsules. In: König H (ed) Prokaryotic cell wall compounds. Springer, Heidelberg, p 111
29. Monack DM, Mueller A, Falkow S (2004) Persistent bacterial infections: the interface of the pathogen and the host immune system. Nat Rev Microbiol 2:747–765
30. Westphal O, Liederitz O, Bister F (1952) Ueber die Extraktion von Bakterien mit Phenol/Wasser. Z Naturforsch. 7B:148–155
31. Pupo E, Aguila A, Santana H, Núñez JF, Castellanos-Serra L, Hardy E (1999) Mice immunization with gel electrophoresis-micropurified bacterial lipopolysaccharides. Electrophoresis 20:458–461
32. Davis MR Jr, Goldberg JB (2012) Purification and visualization of lipopolysaccharide from Gram-negative bacteria by hot aqueous-phenol extraction. J Vis Exp 28:e3916, 1–3
33. Nagy G, Pál T (2008) Lipopolysaccharide: a tool and target in enterobacterial vaccine development. Biol Chem 389:513–520
34. Reisser D, Pance A, Jeannin JF (2002) Mechanisms of the antitumoral effect of lipid
35. Bowden RA, Cloeckaert A, Zygmunt MS, Dubray G (1995) Outer-membrane protein- and rough lipopolysaccharide-specific monoclonal antibodies protect mice against Brucella ovis. J Med Microbiol 43:344–347
36. Fulop M, Mastroeni P, Green M, Titball RW (2001) Role of antibody to lipopolysaccharide in protection against low- and high-virulence strains of Francisella tularensis. Vaccine 19:4465–4472
37. Ada G, Isaacs D (2003) Carbohydrate-protein conjugate vaccines. Clin Microbiol Infect 9:79–85
38. Landsteiner K (1945) The specificity of serological reactions. Harvard University Press, Cambridge
39. Avery OT, Goebel WF (1929) Chemo-immunological studies on conjugated carbohydrate-proteins. II Immunological specificity of synthetic sugar-protein antigens. J Exp Med 50:533–550
40. Pollard AJ, Perrett KP, Beverley PC (2009) Maintaining protection against invasive bacteria with protein-polysaccharide conjugate vaccines. Nat Rev 9:213–220
41. Daum RS, Hohrman D, Rennels MB, Bewley K, Malinoski F, Rothstein E, Reisinger K, Block S, Keyserling H, Steinhoff M (1997) Infant immunization with pneumococcal CRM 197 vaccines: effect of saccharide size on immunogenicity and interactions with simultaneously administered vaccines. J Infect Dis 176:445–455
42. Lefeber DJ, Kamerling JP, Vliegenthart JFG (2001) Synthesis of Streptococcus pneumoniae type 3 neoglycoproteins varying in oligosaccharide chain length, loading, and carrier protein. Chem Eur J 7:4411
43. Paolletti LC, Kasper DL, Michon F, DiFabio J, Jennings HJ, Tosteson TD, Wessels MR (1992) Effects of chain length on the immunogenicity in rabbits of group B Streptococcus type III oligosaccharide-tetanus toxoid conjugates. J Clin Invest 89:203
44. Chernyak A, Kondo S, Wade TK, Meeks MD, Fournier JM, Taylor RK, Kováč P, Wade WF (2002) Induction of protective immunity by synthetic V. cholerae hexasaccharide derived from V. cholerae O1 Ogawa lipopolysaccharide bound to a protein carrier. J Infect Dis 185:950–962
45. Dick WE Jr, Beurret M (1989) A survey and consideration of design and preparation factors. In: Cruse JM, Lewis RE Jr (eds) Glycoconjugates of bacterial carbohydrate antigens, vol 10. Krager, Basel, pp 48–114
46. Tietze LF, Arlt M, Beller M, Glüsenkamp KH, Jähde E, Rajewsky MF (1991) Anticancer agents, 15. Squaric acid diethyl ester: a new coupling reagent for the formation of drug biopolymer conjugates. Synthesis of squaric acid esters amidates and amides. Chem Ber 124:1215–1221
47. Glüsenkamp KH, Drosdzio W, Eberle G, Jähde E, Rajewsky MFZ (1991) Naturforsch C Biosci 46:498–501
48. Tietze LF, Schröter C, Gabius S, Brinck U, Goerlach-Graw A, Gabius HJ (1991) Conjugation of p-amino phenyl glycosides with squaric acid diesters to a carrier protein and the use of the neoglycoprotein in the histochemical detection of lectines. Bioconjuc Chem 2:148–153
49. Cohen S, Cohen SG (1966) Preparation and reactions of derivatives of squaric acid. Alkoxy-, hydroxy-, and aminocyclobutenediones 1. J Am Chem Soc 88:1533–1536
50. Grünefeld J, Bredhauer G, Zinner G (1985) Zur reaktion von quadratsäuredimethylester mit N, N-disubstituierten hydrazin-derivaten. Arch Pharm (Weinheim) 318:984–988
51. Bergh A, Magnusson BG, Ohlsson J, Wellmar U, Nilsson UJ (2001) Didecyl squarate – a practical amino-reactive cross-linking reagent for neoglycoconjugate synthesis. Glycocon J 18:615–621
52. Kamath VP, Diedrich P, Hindsgaul O (1996) Use of diethyl squarate for the coupling of oligosaccharide amines to carrier proteins and characterization of the resulting neoglycoproteins by MALDI-TOF mass spectrometry. Glycocon J 13:315–319
53. Hou S-J, Saksena R, Kováč P (2008) Preparation of glycoconjugates by dialkyl squarate chemistry revisited. Carbohydr Res 343:196–210
54. Saksena R, Adamo R, Kováč P (2007) Immunogens related to the synthetic tetrasaccharide side chain of the Bacillus anthracis exosporium. Bioorg Med Chem 15:4283–4310
55. Bongat AFG, Saksena R, Adamo R, Fujimoto Y, Shiokawa Z, Peterson DC, Fukase K, Vann WF, Kováč P (2010) Multimeric bivalent immunogens from recombinant tetanus toxin HC fragment, synthetic hexasaccharides and a glycopeptide adjuvant. Glycocon J 27:69–77
56. Aebersold R, Mann M (2003) Mass spectrometry-based proteomics. Nature 422:198–207
57. Morelle W, Michalski JC (2005) Glycomics and mass spectrometry. Curr Pharm Des 11:2615–2645
58. Dettmer K, Aronov PA, Hammock BD (2007) Mass spectrometry-based metabolomics. Mass Spectrom Rev 26:51–78
59. Blanksby SJ, Mitchell TW (2010) Advances in mass spectrometry for lipidomics. Annu Rev Anal Chem 3:433–465
60. Banoub JH, Newton RP, Esmans E, Ewing DF, Mackenzie G (2005) Recent developments in mass spectrometry for the characterization of nucleosides, nucleotides, oligonucleotides, and nucleic acids. Chem Rev 105:1869–1915
61. Zhang Y, Go EP, Desaire H (2008) Maximizing coverage of glycosylation heterogeneity in MALDI-MS analysis of glycoproteins with up to 27 glycosylation sites. Anal Chem 80:3144–3158
62. Laštovičková M, Chmelík J, Bobalova J (2009) The combination of simple MALDI matrices for the improvement of intact glycoproteins and glycans analysis. Int J Mass Spectrom 281:82–88
63. Kamath VP, Diedrich P, Hindsgaul O (1996) Use of diethyl squarate for the coupling of oligosaccharide amines to carrier proteins and characterization of the resulting neoglycoproteins by MALDI-TOF mass spectrometry. Glycoconjug J 13:315–319
64. Issaq HJ, Conrads TP, Prieto DA, Tirumalai R, Veenstra TD (2003) SELDI-TOF MS for diagnostic proteomics. Anal Chem 75:148A–155A
65. Liu C (2011) The application of SELDI-TOF-MS in clinical diagnosis of cancers. J Biomed Biotechnol 6:245821
66. Chernyak A, Karavanov A, Ogawa Y, Kováč P (2001) Conjugating oligosaccharides to proteins by squaric acid diester chemistry: rapid monitoring of the progress of conjugation, and recovery of the unused ligand. Carbohydr Res 330:479–486
67. Jahouh F, Saksena R, Aiello D, Napoli A, Sindona G, Kováč P, Banoub JH (2010) Glycation sites in neoglycoconjugates from the terminal monosaccharide antigen of the O-PS of *Vibrio cholerae* O1, serotype Ogawa, and BSA revealed by matrix-assisted laser desorption-ionization tandem mass spectrometry. J Mass Spectrom (10):1148–1159
68. Jahouh F, Saksena R, Kováč P, Banoub JH (2012) Revealing the glycation sites in synthetic neoglycoconjugates formed by conjugation of the antigenic monosaccharide hapten of *Vibrio cholerae* O1 serotype Ogawa with the BSA protein carrier using LC-ESI-QqTOF-MS/MS. J Mass Spectrom 47:890–900
69. Jahouh F, Hou SJ, Kováč P, Banoub JH (2011) Determination of the glycation sites of *Bacillus anthracis* neoglycoconjugate vaccine by MALDI-TOF/TOF-CID-MS/MS and LC-ESI-QqTOF-tandem mass spectrometry. J Mass Spectrom 46:993–1003
70. Jahouh F, Hou SJ, Kováč P, Banoub JH (2012) Determination of glycation sites by tandem mass spectrometry in a synthetic lactose-bovine serum albumin conjugate, a vaccine model prepared by dialkyl squarate chemistry. Rapid Commun Mass Spectrom 26:749–758
71. Jahouh F, Xu P, Vann WF, Kováč P, Banoub JH (2013) Mapping the glycation sites in the neoglycoconjugate from hexasaccharide antigen of *Vibrio cholerae*, serotype Ogawa and the recombinant tetanus toxin C-fragment carrier. J Mass Spectrom 48:1083–1090
72. McCarthy PC, Saksena R, Peterson DC, Lee CH, An Y, Cipollo JF, Vann WF (2013) Chemoenzymatic synthesis of immunogenic meningococcal group C polysialic acid-tetanus Hc fragment glycoconjugates. Glycoconjug J 30:857–870
73. Rostostorf P, Fohlman J (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. Biol Mass Spectrom 11:601
74. Johnson RS, Martin SA, Biemann K, Stults JT, Watson JT (1987) Novel fragmentation process of peptides by collision-induced decomposition in a tandem mass spectrometer: differentiation of leucine and isoleucine. Anal Chem 59:2621–2625
75. Domon B, Costello C (1988) A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates. Glycoconjug J 5:397–409
76. Mock M, Fouet A (2001) Anthrax. Annu Rev Microbiol 55:647–671
77. Pries FG (1993) In: Sonenshein AL, Hoch JA, Losick R (eds) *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular biology. American Society for Microbiology, Washington, DC, p 3
78. Nicholson WL, Munakata N, Horneck G, Melosh HJ, Setlow P (2000) Resistance of Bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev 64:548–572

79. Boutiba-Ben Boubaker I, Ben Redjeb S (2001) Bacillus anthracis: causative agent of anthrax. Tunis Med 79:642–646

80. Read TD, Salzberg SL, Pop M, Shumway M, Umayam L, Jiang L, Holtzapple E, Busch JD, Smith KL, Schupp JM, Solomon D, Keim P, Fraser CM (2002) Comparative genome sequencing for discovery of novel polymorphisms in Bacillus anthracis. Science 296:2028–2033

81. Turnbull PCB (1999) Definitive identification of Bacillus anthracis - a review. J Appl Microbiol 87:237–240

82. Reed LJ, Muench H (1938) A simple method for estimating fifty percent endpoints. Am J Hyg 27:493–497

83. Hoffmaster AR, Fitzgerald CC, Ribot E, Mayer LW, Popovic T (2002) Molecular subtyping of Bacillus anthracis and the 2001 bioterrorism-associated anthrax outbreak, United States. Emerg Infect Dis 8:1111–1116

84. Chun J-H, Hong K-J, Cha SH, Cho M-H, Lee KJ, Jeong DH, Yoo C-K, Rhie G-e (2012) Complete genome sequence of Bacillus anthracis H9401, an isolate from a Korean patient with anthrax. J Bacteriol 194:4116–4117

85. Williams DD, Benedek O, Turnbough CL Jr (2003) Species-specific peptide ligands for the detection of Bacillus anthracis spores. Appl Environ Microbiol 69:6288–6293

86. Chabot DJ, Scorpio A, Tobery SA, Little SF, Norris SL, Friedlander AM (2004) Anthrax capsule vaccine protects against experimental infection. Vaccine 23:43–47

87. Daubenspeck JM, Zeng H, Chen P, Dong S, Steichen CT, Krishna NR, Pritchard DG Jr, Turnbough CL (2004) Novel oligosaccharide side chains of the collagen-like region of BclA, the major glycoprotein of the Bacillus anthracis exosporium. J Biol Chem 279:30945–30953

88. Burkitt WJ, Giannakopoulos AE, Sideridou F, Bashir S, Derrick PJ (2003) Discrimination effects in MALDI-MS of mixtures of peptides-analysis of the proteome. Aust J Chem 56:369–377

89. Kratzer R, Eckerskorn C, Karas M, Lottspeich F (1998) Suppression effects in enzymatic peptide ladder sequencing using ultraviolet – matrix assisted laser desorption/ionization – mass spectrometry. Electrophoresis 19:1910–1919