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Vaccines as physically and chemically well-defined pharmaceutical dosage forms

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Although the identification and development of highly immunogenic antigens often seems to be the key step in the creation of new and more effective vaccines, their formulation into stable prophylactic medicines, often in the presence of adjuvants to boost immune responses, can be an equally important challenge. This transformation of molecules into medicines is, however, often a difficult process due to the molecular complexity of most vaccines as well as some controversy over the degree to which vaccines can be treated as physiochemical entities. Currently, many vaccines are still primarily defined in laboratory tests by their ability to produce immune responses in test animals such as mice. Such responses are most often detected as increases in antibody titers or changes in cytokine production. Some of the drawbacks with such assays include their lack of both precision and accuracy, as well as their labor-intensive, time-consuming and costly nature. Thus, significant physicochemical changes in an antigen's primary, secondary or tertiary structure, such as those seen as losses of stability, often cannot be detected by such studies. Furthermore, no direct evidence is provided concerning the mechanism of inactivation involved in stability alterations, making improvements in stability a primarily empirical process. A potential solution to this problem is to treat vaccines as well-defined physicochemical substances in which particular structural changes can be detected and then used as a basis for formulation alterations to reduce or eliminate deleterious events. The question we address in this article is whether this more well-defined, physicochemical approach to vaccine formulation development is actually possible given currently available technology.

When we think of the structure of a vaccine antigen, such as a protein, plasmid or viral particle, most of us picture an array of atoms arranged in a particular 3D configuration. Modern methods of x-ray crystallography, cryo-electron microscopy and multi-dimensional nuclear magnetic resonance (NMR) spectroscopy produce data that can often be used to develop 3D images of this type. It is natural to assume that structural information of this nature will be necessary to provide the kind of high-resolution picture required to assure the integrity of complex molecular entities such as vaccines. A major problem, however, is that such high-resolution methods are generally applicable to macromolecules and viruses only in either concentrated solutions or dried crystalline states. While the future may indeed involve the extensive use of such methods, their current applicability to vaccines remains technically difficult with low practical utility.

Another possible way to develop a higher resolution approach to the characterization of vaccine antigens might be to think in terms of information theory. Most estimates suggest the information content of proteins ranges from 2 to 3 bits per amino acid residue. Thus, in this view, proteins and other macromolecular antigens are very information rich, and this suggests a corresponding need for experimental techniques that detect a wide range of structural features. It has often been noted, however, that various forms of redundancy substantially reduce the desired information content. Unfortunately, previous

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efforts have been almost entirely based on sequence information and fail to deal either with all of the critical 3D structures or post-translational modifications. Thus, defining the exact nature of the different types of information (and corresponding experimental techniques) necessary to define something like a vaccine antigen to the needed degree of definition to ensure structural integrity remains elusive in terms of practical applications.

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We propose that a third approach, more empirical and pragmatic in nature, is already available that may be able to accomplish higher resolution vaccine antigen characterization and detection of physicochemical structural alterations to substantially reduce our reliance on biological methods. The basic idea is to measure and characterize vaccines by a series of methods that monitor various aspects of structure as a function of multiple stress variables. In terms of the conformational aspects of macromolecular behavior, methods such as circular dichroism (CD) and fourier transform infrared spectroscopy (FTIR) can detect changes in the secondary structure of proteins, nucleic acids and their complexes on the order of 2–3% or less. Aspects of the tertiary structure can be similarly monitored by techniques such as intrinsic fluorescence, near-UV CD and near-UV absorbance spectroscopies. The latter two methods can typically provide five to seven distinct peaks using derivative or other deconvolution algorithms, providing a rich source of tertiary structure information. A number of fluorescent, absorbance and induced CD extrinsic probes can also be used to detect a wide variety of different aspects of structure. Changes in size (dissociation/association of complexes and/or aggregation of antigens) can be analyzed by methods such as size exclusion chromatography, analytical ultracentrifugation, light scattering and digital imaging technologies, among many others. The covalent structure of proteins is now routinely analyzed by HPLC-MS usually employing enzymatic fragmentation (peptide mapping). This permits degradation events such as oxidation and deamidation of amino acid residues to be accurately accessed. Similar methods exist for nucleic acids. The combination of the protein and nucleic acid-based methodologies can be applied to viral vaccines that contain both components.

An additional key consideration for the characterization of any large macromolecular antigen is its more dynamic aspects. It has been recognized for some time that proteins, nucleic acids and lipid membranes can undergo a wide variety of internal motions, ranging from both partial and free rotations of chemical groups around covalent bonds to large movements of structurally defined domains relative to one another. Thus, proteins and nucleic acids are often described in terms of an overall distribution of conformational states. The dynamic nature of such systems is thought to play a critical role in both their stability and biological activity and should therefore be taken into account in any analysis of their behavior. Again, an extensive range of methods are available for this purpose. These include NMR and hydrogen/deuterium isotope exchange, although these approaches tend to be low throughput and currently of limited applicability despite their high resolution. By contrast, higher throughput but lower resolution methods such as ultrasonic spectroscopy, pressure perturbation differential scanning calorimetry, various forms of fluorescence (time-resolved anisotropy, red-edge excitation, solute quenching, and so on) and UV absorbance (cation-pi probing and temperature-dependent peak shift analysis) can be used to better characterize the solution dynamics of macromolecules.

To obtain the maximum amount of information, all of the aforementioned methods can be performed as a function of stress variables such as temperature, pH, concentration, ionic strength, agitation and freeze/thaw events. Such experiments generally have the ability to detect the 'weak spots' in a structure that can lead to a loss of biological activity or immunogenicity under relevant conditions such as long-term storage. One significant problem is the efficient and productive analysis of such a large quantity of physicochemical data. An approach we have used involves the creation of what we refer to as empirical phase diagrams (EPDs). In this version of singular value decomposition (a common method used to decompose matrices in linear algebra), a molecule or molecular complex of interest can be represented as a vector in a highly dimensional experimental space. For example, at any particular combination of stress, variables such as temperature and pH, normalized values of various experimental measurements (e.g., CD for secondary structure, fluorescence for tertiary structure, light scattering for aggregation, and so on) are used to construct a vector that is converted to a color-based scheme (red, green, blue or RGB) for visual representation. A plot of these colored vectors as a function (for example) of temperature and pH then provides a pictorial stress–response picture of protein structure. Such EPDs can be either visually or mathematically compared to provide a detailed comparison of different macromolecular systems such as different vaccine antigens. One can expect new versions of related approaches utilizing newer methodologies of increasingly higher sensitivity and resolution in the near future.

With the realization that dynamic aspects of protein behavior also play a key role in their biological activity and stability, similar EPDs as a function of temperature and pH have been developed utilizing ultrasonic spectroscopy and pressure perturbation differential scanning calorimetry. For the analysis of vaccine antigens, it will become necessary to begin to take into account such phenomena. As described earlier, a variety of methods are available for this purpose, each sensitive to various aspects of molecular motion, and thus providing an even more detailed picture of such systems.

From a vaccine perspective, it is especially important to ask whether such an approach might be applied to a complex entity such as a virus-like particle, virus, protein conjugate, DNA/carrier complex or even a bacterial cell? We think the answer is a qualified yes. Consider, for example, a virus, either live (attenuated) or killed. The actual weight composition of such a particle often tends to be dominated by only one or few proteins, nucleic acid and/or lipid components. Thus, measurements such as CD and

fluorescence (including that of dyes that bind in a conformationally specific manner to a particular component) are often simpler than might be anticipated. To the extent that changes in this component(s) are responsible for activity losses, EPDs can still be usefully applied. This is even more the case with recombinant protein- or DNA-based vaccines, even when multiple species are present as in combination-type vaccines (although an initial separation would obviously facilitate such an analysis).

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What about the more problematic situation of characterization of a live-attenuated viral vaccine? Such formulations often consist of mixture of both live and inactive viral particles. Alterations seen (and potentially measured) in both types of viruses can potentially still be used for formulation development as well as stability studies. While no absolute statements can be made regarding the applicability of physical studies to more complex live virus vaccines, a number of recent studies suggest this may well be possible. While still somewhat technically difficult, the creation of chemical maps now seems within reach of combinations of multidimensional chromatography (e.g., reversed phase/ion-exchange) and high-resolution mass spectrometry. One problem would seem to be how to account for the analysis of minor components as might be present in a viral preparation, but this problem appears to be addressable by appropriate chromatography at the front end. Furthermore, 100% coverage of sequences may not be necessary.

Another important challenge to the characterization of vaccine antigens is the presence of additional substances in vaccine formulations such as adjuvants. The most commonly employed adjuvants are the aluminum salts, which constitute a highly opaque suspension of micron-sized particles to which many protein antigens, virus-like particles and inactivated viruses are typically absorbed. For analysis purposes, antigens can sometimes be removed by use of pH, salt, chaotropic agents or dissolution of the adjuvant. This approach is often problematic, however, since the structure of the antigen may be altered by the desorption process. Recent work has shown that the structure of antigens can be directly characterized on aluminum salt surfaces by methods such as fluorescence, infrared spectroscopy and differential scanning calorimetry. While such work should still be considered in its infancy, it seems highly likely that the presence of adjuvants, including newer candidates such as lipid A, QS-21, CpG oligonucleotides and even microemulsions, will not represent an insurmountable obstacle to the empirical approach.

We believe that the key step to developing an improved physicochemical understanding of vaccines is the adoption of a pragmatic approach as described in this article. By this, we simply mean that a series of lower resolution analytical procedures yields a 'true' picture of the vaccine of interest to the extent that it 'works' in a practical sense. For such an approach to reach the state of pharmaceutical utility, ongoing collaborations between industrial, government and academic laboratories will be necessary to further develop our understanding of the inter-relationships of a vaccine's structural integrity and biological activity. Importantly, ongoing evaluation of results from these physicochemical measurements as applied to a wide variety of vaccines, in terms of successes or failures to understand and enhance vaccine stability, will provide increasing confidence in this pharmaceutical approach to developing vaccine dosage forms. The result should be the more rational design of vaccine formulations with greatly improved stability and efficacy.

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