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INVITED REVIEW

Peptides for immunological purposes: design, strategies and applications

Alessandro Gori · Renato Longhi · Claudio Peri · Giorgio Colombo

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Abstract The development of new vaccines remains an attractive goal for disease prevention and therapy, in combination or alternative to drug-based treatment. In parallel, a growing awareness of the importance of early diagnosis in successful disease management is driving the demand for new reliable diagnostic tools. As a consequence, over the last decades an impressive amount of work has been directed toward the search for new solutions to address vaccine design and biomarker discovery. In this context, peptides have generated considerable interest thanks to their general accessibility and ease of manipulation. The aim of this review is to provide the reader a general picture of the traditional peptide-based strategies adopted in immunology and to report on recent advances made in this field, highlighting advantages and limitations of classic versus innovative approaches. Case studies are described to provide illustrative examples, and cross references to more topic-focused and exhaustive reviews are proposed throughout the text.

Keywords Antigenic peptides · In silico epitope predictions · Vaccine development · Biomarker discovery · Structural vaccinology · Molecular design

General concepts to epitope prediction and design

Disclosing the molecular mechanisms that drive biologically relevant processes is a fundamental goal for biological chemistry. Understanding how biomolecules interact, unveiling which key interactions are involved in cellular pathways and revealing which of them are misregulated in the development of a pathological condition may help defining new targets for pharmacological intervention. As proteins modulate the majority of biological functions, exploiting structural information on how protein–protein interactions (PPIs) are controlled at the atomic level can pave the way toward their rational manipulation. In this context, the popularity of peptides as modulators/inhibitors of PPIs has consistently grown over the last few years, thanks to the synthetic accessibility and flexible application potential of these molecules. For instance, peptides and peptidomimetics with different conformational dynamic properties and designed structural preferences have been used to probe the role of conformational selection and preorganization in regulating key interactions involving proteins (Bock et al. 2013; Chatterjee et al. 2008; Goodman et al. 2007; Haridas 2009; Pedersen and Abell 2011; Souroujon and Mochly-Rosen 1998). The amount of knowledge generated by experimentally determined sequence–structure–function relationships contributed to lay the foundations for computational design methods. In general, computational peptide or protein design entails the search for peptidic sequences and amino acids that adopt defined structures and functions (Floris and Moro 2012; Fung et al. 2008; Nikiforovich 2009; Renfrew et al. 2012; Yin et al. 2007). Computational methods have further evolved into the re-design of protein–protein interfaces. The work of Kortemme (Mandell and Kortemme 2009; Smith and Kortemme 2010), Kuhlman (Der et al. 2012; Lewis and Kuhlman 2011; Sammond et al. 2011), Baker (Fleishman et al. 2011; Koga et al. 2012; Richter et al. 2012) provides elegant examples of how interfaces can be redesigned to modulate in vitro functions. These efforts

A. Gori · R. Longhi · C. Peri · G. Colombo (✉)
Istituto di Chimica del Riconoscimento Molecolare, Consiglio Nazionale delle Ricerche, Via Mario Bianco 9, 20131 Milan, Italy
e-mail: g.colombo@icrm.cnr.it

brought about the realization that functional protein/peptide interactions are determined by a well-balanced interplay of sequence, physico-chemical, structural and conformational properties. As a consequence, predictions of protein interaction properties aimed at supporting the design of functional molecules should take into account not only sequence and structural similarities or homologies, but also the dynamic and energetic properties that occur at protein interfaces (Hoffman et al. 2005; Keskin 2007; Sheinerman et al. 2000; Ulucan et al. 2012; Zen et al. 2010).

Among other fields, these concepts find application in immunology where considerable effort has been put into developing platforms to screen candidate protein antigens and to identify/predict minimal antigenic regions (epitopes) responsible for immunoreactivity. In particular, a lot of interest has been conveyed toward the antibody-mediated (humoral) components of the immune system since, due to their peculiar role and activity, antibodies can represent valuable tools for both therapy and diagnosis (Peters 2000; Waldmann 1991). Indeed, the identification of reactive antigen regions that are determinant for antibody elicitation and recognition still represents an attractive and challenging opportunity for predictive rational-based computational methods. Epitopes are conventionally classified as continuous, i.e., sequential and relatively short peptides from the protein sequence able to bind anti-antigen antibodies, or discontinuous, i.e., patch of atoms/fragments from not-contiguous protein regions which are brought to close proximity by protein folding and whose antigenicity depends upon the protein conformation. It is worth underlining that epitopes do not exist as discrete and structured entities, but rather have fuzzy boundaries and are defined by their functional ability to bind antibodies (Van Regenmortel 2009a). While continuous epitopes can in theory be predicted out of the protein sequence by consensus of available datasets of immunogenic peptides (Greenbaum et al. 2007), the prediction of discontinuous epitopes relies upon the knowledge of the protein 3D structure. Over the last few decades, several approaches have been tested to run epitope prediction from protein structure. Some groups attempted to correlate antigenicity with protein region properties such as solvent accessibility or flexibility (Novotný et al. 1986; Westhof et al. 1984). For instance, electrostatic desolvation profiles (EDP) method hypothesizes that surface protein regions with a small free energy penalty for water removal may correspond to preferred interaction sites and may, accordingly, in the case of antigens, constitute binding sites for antibodies (Fiorucci and Zacharias 2010). Others based their approach on identifying those regions that protrude out of the protein globular surface and relating them to antigenicity (Ponomarenko et al. 2008; Thornton et al. 1986). In a reverse approach, Molina and colleagues targeted the identification of epitopes within the protein structure through a bioinformatic analysis of sets of mimitope sequences, i.e., randomly generated peptides mimicking epitopes functional antibody recognition properties (Moreau et al. 2006). By integrating mimitopes' alignments and consensus, the authors were able to identify the original epitope regions targeted by antibodies within the native antigen in a series of case models where antibody–antigen crystal structures were available. In this direction, our group has recently developed a new way to approach in silico epitope prediction based on the integrated analysis of the dynamical and energetic properties of an antigen, namely matrix of local coupling energies (MLCE) (Scarabelli et al. 2010). The key assumption of MLCE method is that epitopes may correspond to protein regions that are not involved in stabilizing interactions within the protein fold, since they must continuously evolve to escape recognition by the immune system without affecting protein functional structure. To be recognized by a (antibody) binding partner, these regions should also well tolerate conformational changes with minimal energetic expense. Accordingly, MLCE prediction is based on antigen structure analysis identifying those localized regions that are less energetically coupled with the rest of the protein, and that consequentially fit basic criteria for representing epitope candidates. To date, the MLCE method found successful application in epitopes prediction for proteins FABP3 and S100B (Peri et al. 2013) and for the discovery of biomarker and vaccine candidates for the *Burkholderia Pseudomallei* pathogen (Lassaux et al. 2013). MCLE and EDP methods, along with many others, are intrinsically limited to predict regions exposed on the protein surface. As a methodological evolution, in silico dissection of proteins into domains prior to epitope prediction may expose low-accessibility regions that may be targeted by antibodies under conditions of partial unfolding/degradation, thus improving prediction performances (Genoni et al. 2012). By applying this strategy, we indeed showed an improved match between theoretical and experimental epitope mapping (Lassaux et al. 2013).

A landmark integration of atomic-resolution information with computational techniques was reported by Schief and collaborators working on a hybrid method for the grafting of functional motifs onto unrelated protein scaffolds to accurately replicate the antigenic surface recognized by target antibodies (Azoitei et al. 2011; Correia et al. 2010; Ofek et al. 2010). Epitope scaffolding strategy, by mimicking complex antigenic targets, might be particularly useful to reproduce discontinuous epitopes and enhance the extent of the immune response against antigens for which elicitation of antibodies has been demonstrated to be particularly challenging (e.g., transient, immunorecessive,

cryptic). Indeed, the scaffold-supported antigen is 'presented' in a context that lacks pathogen defensive mechanisms that have evolved to elude the immune response (Burton 2010). In one case example, the authors' work focused on transplanting a two-segment discontinuous HIV gp120 epitope on a suitable scaffold that may accommodate the entering motif without altering its original functional conformation (Azoitei et al. 2011). Scaffold selection and design for optimal motif transplantation were computationally assisted, as well as the generation of a small set of mutagenesis libraries to undergo functional screening. The authors were able to generate a scaffold-bound motif displaying specificity and affinity for antibody recognition similar to the original gp120. This strategy may be then potentially suitable for using grafted epitopes as immunogens to elicit neutralizing antibodies.

Overall, many different approaches have been exploited toward epitope identification, prediction and design. The need to overcome limits associated to mere (time consuming) experimental methods has strongly oriented research in this area toward computationally assisted schemes, where information from experimental evidence is merged with in silico methodologies to investigate protein 'interactome'. As general knowledge is progressing, new perspectives and solutions are expected to come to light. The generation of more and more reliable methods to approach complex models might then represent a closer step toward the full realization of rational-based manipulation of PPIs. In this scenario, the fast prediction and successful design of epitope candidates aimed to finalize their therapeutical/diagnostic application is not out of reach.

Peptides as vaccine candidates

Despite the progresses made in the field of drug discovery, vaccination still remains an inescapable approach in modern medicine. Indeed, the increasing incidence of drug resistance, which conventional antibiotic therapies are facing, poses a serious threat to maintaining the current public health status (Loddenkemper and Hauer 2010; Nikaido 2009). In addition, a strong demand for vaccine development is arising for the prevention of noninfectious diseases such as cancer, for which immunotherapy may represent a valid alternative/adjuvant to pharmacological treatment (Sharav et al. 2007; Tsunoda 2004). Last but not least, the access to prolonged and expensive drug-based therapies still remains a major issue in the less developed countries, making disease prevention more desirable. Vaccines have traditionally consisted of attenuated or inactivated microorganisms delivered by injection. However, since a series of inherent practical issues is associated to this approach along with increasing safety demands from regulation authorities, research in the vaccine field has witnessed a growing interest toward attempts to develop protein- and peptide-based vaccine candidates (Purcell et al. 2007). In this context, reverse vaccinology (RV) (Masignani et al. 2002) and structure-based antigen design (Dormitzer et al. 2008), a.k.a. structural vaccinology (SV) techniques, have generated high expectations toward a 'new era' of fast and safe vaccine development (Fig. 1). RV encompasses in silico pathogen genome analysis aimed to identify those protein antigens that, among all, are most likely to represent vaccine candidates (e.g., cell-surface exposure, protein stability). Candidate identification disregards traditional limitations such as whether an antigen is abundantly or modestly expressed, whether it is constantly or temporarily expressed and whether it is easily isolated or not. Virtually, all genes of a pathogen can be screened, enabling this platform to generate vaccine candidates in a fast, safe and effective way even for pathogens against which classical approaches have failed. With regard to SV, the approach relies on the concept that by knowing which parts of an antigen are effectively responsible for antigenicity, it is possible to engineer the native antigen to improve its feasibility as vaccine candidate. In particular, identifying those components within the antigen structure that elicit protecting immunity allows performing antigen manipulation driven by structural considerations (e.g., domain stabilization, conformational constraints). In this way, efforts toward vaccine optimization can be focused on those antigen regions that play a significant role in immunity. As a further development of the SV approach, it may then be viable to select only the antigen regions able to elicit an immune response and translate them in the form of peptides as potential vaccine candidates. Although often underestimated, there are several advantages to peptide-based vaccine approaches over traditional ones, ranging from avoiding the injection of infectious and potentially harmful material to the ease of synthesis, manipulation and storage with respect to in vitro culture and handling of dangerous pathogens and to the possibility of focusing on the use of minimal immunogenic regions of a protein antigen (Purcell et al. 2007). While very promising, regardless of the considerable efforts, the full realization of peptide-based approach toward vaccine development is still to be fulfilled (Van Regenmortel 2009b). Nevertheless, the search for new strategies to deliver peptide vaccines remains vivid, as potential advantages may overcome current limitations.

The goal of vaccination is to stimulate an antigen-specific response, possibly involving both the cellular (CD8+ cytotoxic and CD4+ helper T cells) and humoral (antibody) components of the immune system. The antigen specificity resides in its minimal regions that are responsible for

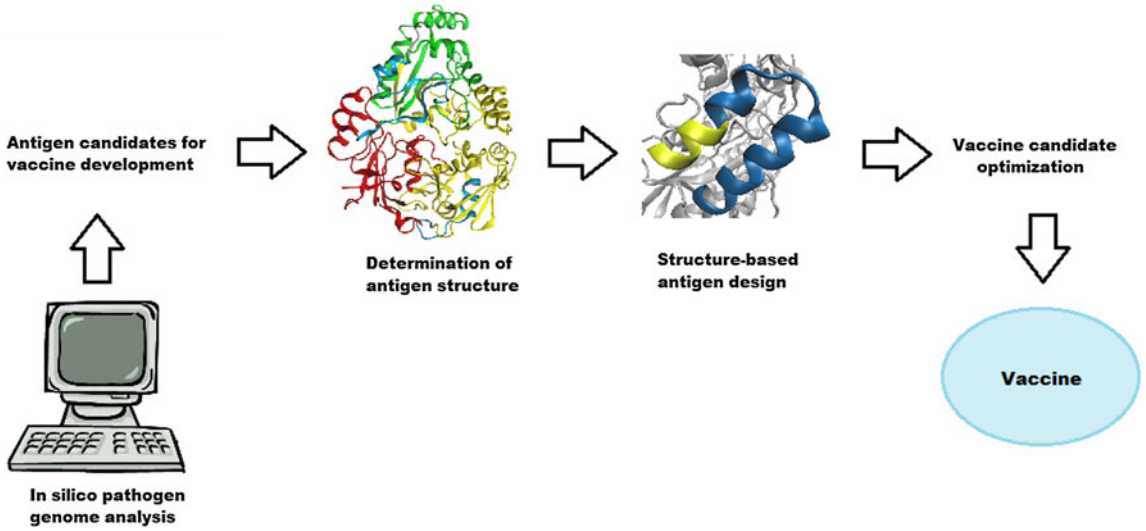

Fig. 1 Flowchart for RV and SV approaches toward vaccine development. Computational analysis of pathogen genome identifies those antigens that present ideal features for vaccine development. Following structure determination, antigen can directly undergo optimization or constitute the basis for the identification of its antigenic regions. Epitopes can in turn undergo optimization to best display their immunogenic potential

immunoreactivity, namely epitopes. Due to the different mechanism by which humoral and cellular responses are generated (Fig. 2), requirements for peptide epitope design differ substantially. As the T cell-mediated response follows a complex intracellular antigen processing pathway, only little conformational requirements, if any, have to be taken into account in designing epitopes to induce a CTL-mediated response (Purcell et al. 2007). On the contrary, to elicit a specific antibody response that recognizes the parent antigen, a peptide epitope should in principle trace the conformational properties of the native antigenic region. Unfortunately, only in few cases short peptides extracted out of their original context possess a conformation that mimics the one they adopt within the native antigen (Van Regenmortel 2009b). Thus, despite that in any case an antibody response might be generated against a peptide epitope, antibodies might not be cross-reactive toward the parent antigen. Consequently, unless the peptide used for immunization mimics or is forced to mimic the conformation of the corresponding native antigenic region, elicited antibodies are unlikely to cross-react specifically with the parent protein. Several approaches are available to induce a correct folding of free peptides, but previous knowledge of the whole antigen structure is therefore necessary (Purcell et al. 2003). Moreover, the fact that most protein epitopes are actually discontinuous, i.e., are formed by groups of atoms belonging to residues located on not-contiguous protein segments that are brought together by the peptide chain folding, is often underestimated (Van Regenmortel 2009a). Thus, it is not surprising that most continuous epitopes are not able to elicit really neutralizing antibodies. In recent years, the work of some groups has focused on attempts to reproduce complex discontinuous epitopes by means of synthetic constructs. This approach is usually based on anchoring different peptide sequences to synthetic scaffolds, to contemporarily block peptide conformations and bring in close contact sequences which are assumed to be part of a discontinuous epitope. In this context, it is worth citing the work by Liskamp and collaborators on antigen surface mimicking by the use of a triazacyclophane (TAC) scaffold (Fig. 3a); (Hijnen et al. 2007; Mul der et al. 2012), as well as the functional reconstruction of conformational epitopes by means of the CLIPS technology (Chemical Linkage of Peptides onto Scaffolds) reported by (Fig. 3b) Timmerman et al. (2007, 2009). While the potential of these strategies is still to be fully demonstrated, this kind of approach appears to be promising. As a further complication, inherent limitations to peptide-based vaccination come from the small size of peptide molecules and the low copy number of peptide-based immunogens, along with their poor immunogenicity when administered in the absence of a vaccine adjuvant (Hervé et al. 1997; Van Regenmortel 2001). Traditionally, these problems have been addressed by the conjugation of peptides to protein carriers (BSA, KLH, OVA, etc.) to serve both as delivery systems for multiple epitopes display and as T-helper epitopes source for response amplification (Chiarella et al. 2010). Despite this strategy being widely used, it still suffers from poor homogeneity of peptide-carrier adduct composition and of possible alterations of epitope integrity following conjugation (Briand et al. 1985). Moreover, carrier-induced antigen suppression, i.e., induction of an antibody response against the carrier to a much greater extent than toward the epitope can occur (Herzenberg et al. 1980; Schutze et al.

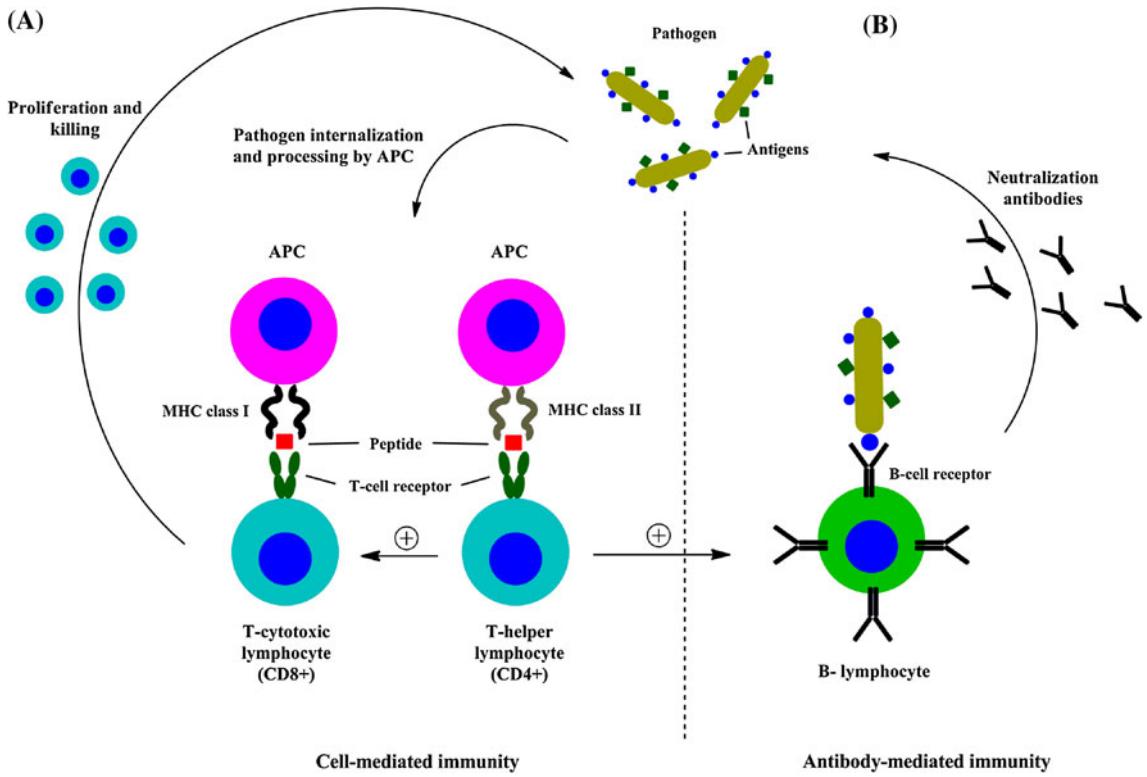

Fig. 2 Cellular- and humoral-mediated components of the immune response. **a** In order to activate CTL-mediated response, antigens must first undergo cellular internalization by APCs followed by a complex proteolytic processing pathway. The resulting T-cell epitopes binding major histocompatiblity complex class I (MHC I) are presented for recognition by T-cell receptor. Mutual recognition, along with interaction with T helper cells, triggers activation and proliferation of naïve CD8+ T cells to generate a killing response. **b** Upon immunogen recognition and internalization mediated by B-cell receptor (immunoglobulin receptor), and following activating interplay with T-helper lymphocytes, B cells differentiate into plasma cells that secrete antibodies with identical specificity of the immunoglobulin receptor. Other co-stimulatory molecules are involved in each step recognition, as well as cytokine secretion plays a deep role in determining the type of response that is generated

1985). Multiple antigen display was elegantly pioneered by Tam and co-workers, who first introduced the concept of multiple antigen presentation (MAP) by use of branched oligolysine constructs bearing multiple epitopes (Francis et al. 1991; Huang et al. 1994). Since then, many groups have reported on the advantages of branched over linear peptides as better immunogens (Fitzmaurice et al. 2000; Rosenthal 2005; Schott et al. 1996), paving the way to the search for alternative strategies to combine multiple epitopes in a controlled way. For instance, Jackson and collaborators conceived the synthesis of multi-peptide polymers, by introducing an N-terminal acrylated residue to single peptide epitopes followed by radical-free polymerization to generate a covalently bound platform displaying several copies of the same epitope/different epitopes (Brien-simpson et al. 1997; Jackson et al. 1997; Sadler et al. 2002). Others explored the use of dendrimers (Kowalczyk et al. 2010). More in general, with the progressive development of chemoselective synthetic and conjugation techniques, many strategies have been applied to multiple antigen presentation (Fujita and Taguchi 2011). One critical aspect in the development of peptide vaccines is their low immunogenicity as single entities, as a consequence of their weak activation of antigen-presenting cells (APC) such as dendritic cells (DC) and macrophages, which in turn play a key role in activating B and T lymphocytes. Thus, conventional peptide vaccine formulations usually include adjuvants (e.g., complete Freud's adjuvant, CFA) to favor the onset of an immune response (Wang 2011). However, most of the adjuvants commonly used in animal vaccination protocols are toxic and therefore not suitable for human administration. The synthesis of platforms bearing both peptide epitopes and a lipophilic moiety is now considered a valuable way to produce self-adjuvanting constructs that allow overcoming intrinsic low peptide immunogenicity (Moyle and Toth 2008). Indeed, bacterial lipid components such as tripalmitoyl-S-glyceryl cysteine (Pam3Cys) and Pam2Cys are known agonists of the Toll-like receptors (TLRs) family, a class of receptors expressed on the surface of a wide variety of cells including APC and whose activation is a key step in the generation of an immune response (Jackson et al. 2004; Zhu et al. 2004). Therefore,

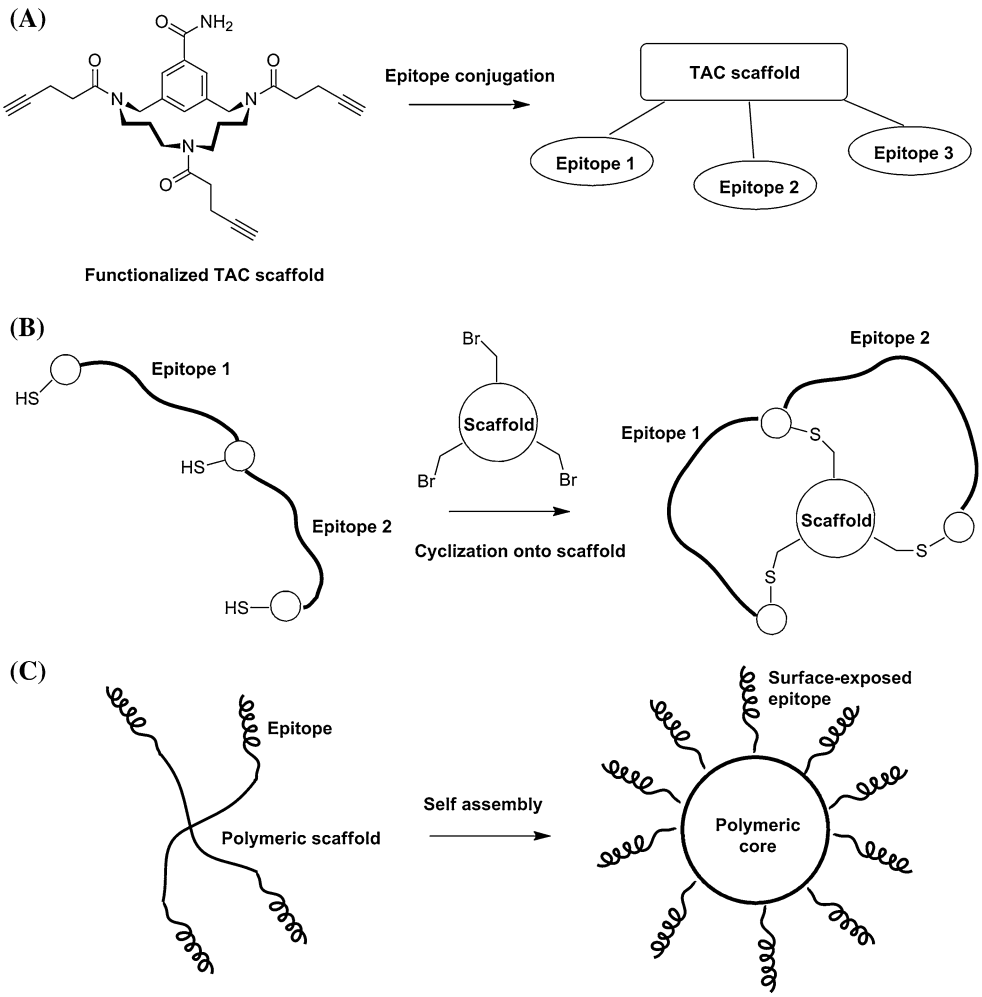

Fig. 3 **a** TAC scaffold-mediated mimicking of antigen surface. Different segments of discontinuous epitopes are anchored to one functionalized scaffold. Once brought in close proximity (*cyclic*), sequences can provide a good mimicking of the situation found in the discontinuous epitope. **b** Schematic representation of CLIPS technology; different regions of discontinuous epitopes are synthesized as one construct and then undergo cysteine-mediated cyclization onto a scaffold to provide a functional mimetic of the native antigenic region. **c** Synthesis of dendrimeric epitope platform that self-assembles into nanoparticles. Polymer dendrimer forms nanoparticle core, while epitope remains exposed on particle surface

lipopeptides have found broad application as candidates for vaccine development, since in addition to immunogenicity enhancement none of the side effects associated with the use of adjuvants is usually encountered (Galdiero et al. 2012; Zeng et al. 2002). Similarly, proteins/peptides glycosylation is known to play an important role in immunity, especially in the T-cell stimulation, as carbohydrates act facilitating the cellular uptake through a receptor-mediated internalization (e.g., mannose receptors) (Rudd et al. 1999, 2004; Rudd 2001). It is then not surprising that a deep interest in glycopeptides has been found in the field of synthetic vaccine candidates (Bay et al. 1997; Fujita et al. 2008; Kowalczyk et al. 2012).

In recent years, the field of vaccine development has experienced a growing awareness of the importance of the way through which an antigen is presented to fully 'realize' its immunogenic potential. Particularly, considerable interest has been shown in the use of nanoparticles as vaccine delivery systems (Akagi et al. 2012; Foster et al. 2010; Uto et al. 2009; Yoshikawa et al. 2008; Zolnik et al. 2010). Notably, nanoparticles prepared from biocompatible and biodegradable polymers have been proposed not only as mere vaccine carriers, but also as potent immune response adjuvants. Antigen-loaded nanoparticles are indeed claimed to target and activate more specifically APCs, at the same time allowing controlling intracellular antigen release. As further evolution, the interest of many groups is now oriented on the development of peptide-based self-assembling systems, which were shown to be suitable devices to address multiple antigen presentation and self-adjuvation. An elegant explanatory case was reported by Toth and co-workers working on the group A streptococci (GAS) (Skwarczynski et al. 2010). The authors synthesized a polyacrylate dendritic structure that was used as scaffold to attach several copies of a peptide antigen. The whole construct was shown to self-assemble in nanoparticles (20 nm in diameter) possessing a polymeric core and peripherally exposing the antigenic peptide epitope on the particle surface (Fig. 3c). Mice immunization resulted in the production of high titers of antigen-specific antibodies without the need of further adjuvants, validating the initial assumption of a self-adjuvanting system. More recently, Payne and collaborators reported on the design and synthesis of a tricomponent cancer vaccine

candidate that consisted of a glycopeptide or peptide antigen, a universal T-helper peptide epitope and a lipophilic moiety as immunoadjuvant (Wilkinson et al. 2012). The system spontaneously self-assembled to form discrete nanoparticles and was shown to be fully self-adjuvanting and to induce a strong and selective humoral response in murine models. As proofs of concepts of the effectiveness of self-adjuvanting and self-assembling systems keep emerging, the interest toward their further development is expected to grow over the next few years.

In conclusion, a tremendous amount of work has been done so far in the search for a peptide-based vaccine development strategy. Although a truly satisfactory success has yet to be reached, new valuable approaches and strategies to overcome traditional limitations are becoming available. Key considerations driving future development efforts might involve improved antigen candidate selection and design, a better understanding of the role of epitope conformation, a more reliable control over it and the realization of complex but well-defined platforms for peptide antigen display and delivery.

Peptides: tools for biomarker discovery

A biomarker can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Biomarkers definition working group 2001). Over recent years, the increased diffusion of pathologies such as autoimmune diseases and of problems related to pathogen drug resistance, along with sustained efforts in cancer and neurological disorders research, has produced a growing interest in the discovery of new biomarkers as selective tools for disease diagnosis, staging, follow-up and personalization of therapy (Baker 2005; Frank and Hargreaves 2003; Ludwig and Weinstein 2005). It is now widely assumed that an early and timely diagnosis can have a deep impact on disease progression. This is particularly important for those pathologies for which effective therapies are still missing or that are susceptible to misdiagnosis, leading to wrong and potentially harmful treatment. However, biomarkers identification is not always easy, since for many pathologies the molecular causes that trigger disease onset and progression are not known. Furthermore, to be fully reliable a biomarker should be truly specific for a particular biological/pathological status, not leaving space for doubtful interpretation.

In this direction a considerable help comes from the immune system itself, which is 'designed' to selectively recognize the presence of non-self entities and to evoke a protective response against them (Gonzalez et al. 2011; Katsikis et al. 2007). Indeed, an adaptive immune response, either humoral (B cell mediated) and/or cellular (T cell mediated), is usually associated with a pathological status, whether considering cancer, an immune-mediated disease or an infective pathology. Of particular interest to biomarker discovery, the immune system acts amplifying particular antibodies that recognize disease-specific antigens. In this context, the detection of those antibodies in the patient biological fluids that are overproduced in a disease status can represent a valuable diagnostic indicator to drive its clinical management (Anderson and LaBaer 2005). As a consequence, attempts to identify candidate biomarkers have traditionally focused on the screening of putative antigen arrays against easily accessible biological fluids to capture overproduced antibodies which may represent good indicators of a pathogenic condition (Blennow et al. 2010; Robinson et al. 2002). In this scenario, the use of peptide libraries in place of whole antigens may represent a faster, more flexible and easily accessible way to target antibody recognition. A focused approach may be offered by small peptide libraries that are designed to represent only those antigen regions that are likely to be responsible for antigen–antibody interaction, i.e., for immunoreactivity. An example is given by our recent work on melioidosis, an endemic and potentially lethal disease spreading in tropical and subtropical regions, which is often misdiagnosed as tuberculosis and consequently mistreated (How and Liam 2006). Working on a collection of protein antigens of the *B. Pseudomallei* pathogen, melioidosis etiological agent (Wiersinga et al. 2006), we used their 3D structures as the basis for the prediction of potential antibody-binding regions by means of our recently developed MCLE method (Scarabelli et al. 2010). Predictions were then converted in the synthesis of candidate peptides, which were spotted on an ELISA plate and screened for immunoreactivity against sera from healthy donors, and seropositive and seronegative patients. In the case of the OppA antigen (Lassaux et al. 2013), we were able to identify one peptide (COMP3) that showed very interesting discrimination properties, being differently reactive with respect to the three patient groups. Thus, an inherent potential for diagnostic purposes was proven. Notably, COMP3 peptide design was successful out of only three peptides tested, proving the possibility to efficiently connect antigen structure analysis, peptide synthesis and immunoreactivity testing. Working on the same target, a complementary experimental approach based on immunocapturing and proteolytic digestion provided more peptide biomarker candidates (Lassaux et al. 2013). The application of this 'mixed' method is currently ongoing on other *B. Pseudomallei* protein antigens (OmpA, FliC, BPSL 1050, BPSL 0919) with promising preliminary results, especially for the OmpA antigen for whom we were able to

deliver a highly and specific immunoreactive synthetic peptide, which was not only able to distinguish among the three patient groups, but also even recognized to the same extent as the whole recombinant protein antigen. As an obvious limitation, MCLE-based strategy is not suitable for those cases where antigens that trigger primary immune response (primary antigens) or their structure are unknown, and more in general the method is biased by the fact that it may not take into account the wide range of post-translational modifications that can be responsible for the development of a pathological condition, which finds an illustrative example in the case of autoimmune diseases (Lindstrom and Robinson 2011). Autoimmune diseases are in fact usually generated by either native or aberrant post-translational modifications of self-proteins that can affect the tolerance of the immune system toward a protein antigen (neo-antigen) and lead to the development of a self-directed immune response (Anderton 2004; Doyle and Mamula 2001). Partially due to this reason, traditional proteomic-based approaches aimed at biomarkers' identification in autoimmune diseases have often proven unsuccessful. In this case, the use of synthetic peptide libraries that include a wide range of post-translational modifications that occur in proteins, thus generating potential mimetic of the neo-antigens, is surely a valuable approach [for a more extensive review on the topic see (Papini 2009)]. Modifications such as acetylation, glycosylation and citrullination, which cover a part of the possible ones responsible for neo-antigens generation, can indeed be specifically and more easily introduced into peptides with respect to recombinant proteins. This offers a powerful tool to generate candidate biomarkers able to detect autoantibodies in biological fluids. In this strategy, namely 'chemical reverse approach' (Alcaro et al. 2007), the autoantibodies 'select' those modified peptides which resemble the newly generated antigenic regions that trigger the immune response at the origin of the disease. Thus, the chemical reverse approach is in principle able to identify the side-chain modifications effectively responsible for pathology development. After the identification of candidate biomarkers, structure–activity relationship studies can follow to fully characterize the antigen–antibody binding and to lead candidate peptide development toward high specificity of recognition. Examples of this approach validation can be found in the field of systemic lupus erythematosus (Mahler et al. 2005), rheumatoid arthritis (Girbal-Neuhauser et al. 1999; Vossenaar and Van Venrooij 2004) and in a series of papers by Papini and co-workers on multiple sclerosis where a glycopeptide probe for autoantibodies detection was firstly discovered and then developed by an accurate and extensive refinement work (Carotenuto et al. 2001, 2008; Lolli et al. 2005a, b; Mazzucco et al. 1999; Papini 2005). More recently, Kodadek and collaborators tackled biomarker discovery for pathological conditions for which primary antigens are unknown from a new point of view (Reddy et al. 2011). Rather than focusing on attempts to identify the antigen itself or a close mimic to it, they assumed work in a fully unnatural chemical space, i.e., to use combinatorial libraries of unnatural synthetic molecules that may cover a conformational space not represented by unmodified biomolecules. The key concept behind this approach is that, as the primary immune response to some diseases follows an initial marked aberration in a biomolecule, unnatural molecules are more likely to adopt shapes that allow the binding to antibodies generated in response to the original structural modification with respect to naturally occurring molecules, since those shapes cannot be formed by unmodified molecules. Molecules able to bind selectively antibodies elicited against their triggering agent can be seen as antigen surrogates and can display potential for diagnosis. The authors validated their approach by screening libraries of peptoids (N-substituted oligoglycines) against sera from patients with Alzheimer's disease (AD) and were able to identify two candidate antibody biomarkers. Not being focused on mimicking a particular antigen, this approach is claimed to be broadly applicable toward the rapid discovery of antibody biomarkers by high-throughput screening of synthetic molecules.

In summary, even if in recent years great expectations have arisen toward modern protein technologies to drive biomarker discovery, they are still to be fully met. Particularly in the case of autoimmune diseases, cancer and neurological disorders, approaches to new candidate biomarker identification are now shifting from traditional antigen arrays toward the application of new strategies bypassing prior antigen knowledge, similarly to recent schemes in the field of drug discovery. Nevertheless, in cases where antigens are known, new design techniques and rational-driven optimization work may represent an added value that could enlarge discovery possibilities. Given their prompt access and ease of manipulation with respect to more complex antigens, the use of peptides and their mimics or derivatives as fundamental tools to satisfactorily address biomarkers discovery is then expected to keep growing.

Conclusion

Over recent years, the scientific community has been struggling to fully realize the potential and the hopes which the advent of the '-omic era' has brought with it. In particular, the development of new and effective methodologies to fulfill unmet therapeutic needs has witnessed a switch from empirical-based to rationally driven

approaches, a scenario where computational techniques have found fertile ground. The birth of cutting edge methodologies to assist and accelerate data analysis and comprehension of molecular mechanisms involved in pathological conditions is opening the way for new solutions, for therapeutics and diagnostics. In this context, peptide science constitutes an appealing resource, since, in terms of accessibility and flexibility, enormous advantages are potentially associated with the use of peptides and derivatives in place of whole proteins. It is then not surprising that the interest peptides have aroused in the immunology field has considerably grown in recent times. Although a true feasibility of peptides as vaccine candidates that may lead to their wide application is yet to be demonstrated, emerging considerations, along with awareness of current limitations and new strategies to overcome them, keep this prospective alive. Nonetheless, peptides are finding increasing application in the discovery of new biomarkers, which still remains an attractive target to effectively tackle disease management. Following previous considerations, interest in peptide immunology is thus expected to remain vivid in the future, with the hope of finally achieving worthy goals which current efforts are attempting to address.

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Conflict of interest The authors declare that they have no conflict of interest.

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