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Armamentarium of Cryoprotectants in Peptide Vaccines: Mechanistic Insight, Challenges, Opportunities and Future Prospects

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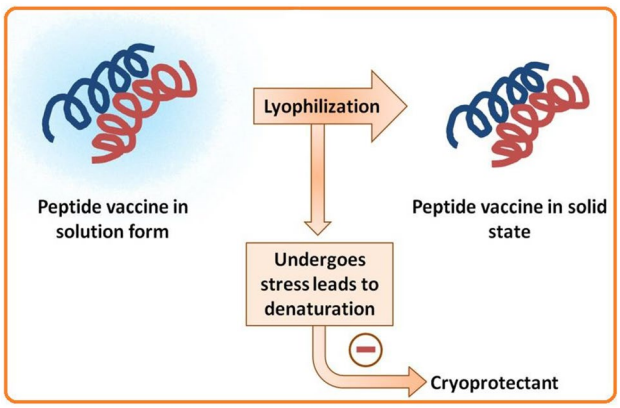
Abstract

Vaccines are designed to leverage the immune system and produce long-lasting protection against specific diseases. Peptide vaccines are regarded as safe and effective way of circumventing problems such as mild allergic reactions associated with conventional vaccines. The biggest challenges associated with formulation of peptide vaccines are stability issues and conformational changes which lead to destruction of their activity when exposed to lyophilization process that may act as stressors. Lyophilization process is aimed at removal of water which involves freezing, primary drying and secondary drying. To safeguard the peptide molecules from such stresses, cryoprotectants are used to offer them viability and structural stability. This paper is an attempt to understand the physicochemical properties of peptide vaccines, mechanism of cryoprotection under the shed of water replacement, water substitution theory and cation-pi interaction theory of amino acids which aims at shielding the peptide from external environment by formation of hydrogen bonds, covalent bonds or cation-pi interaction between cryoprotectant and peptide followed by selection criteria of cryoprotectants and their utility in peptide vaccines development along with challenges and opportunities.

Graphic Abstract



Keywords Synthetic peptide vaccine · Lyophilization · Cryoprotectants · Peptides and proteins

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Abbreviations

BCG	Bacillus Calmette–Guerin
APCs	Antigen presenting cells
MHC	Major histocompatibility complex
DMSO	Dimethyl sulfoxide
T_g	Glass transition temperature
FTIR	Fourier transform infra-red

mAb	Monoclonal antibody
DEAE	Diethylamino ethyl
BSA	Bovine serum albumin
HER2	Human epidermal growth factor receptor 2
LINCS	Linear constraint solver

Introduction

Synthetic peptide vaccines, a recent development in the field of immunology, which are completely synthesised from amino acids (Bijker et al. 2007; Skwarczynski and Toth 2016). The principle of synthetic peptide vaccine involves thorough knowledge of epitope sequences of antigenic determinants of target disease and synthesis of identical peptide chains which are capable of inducing immunity in the host against the disease (Moisa and Kolesanova 2010; Skwarczynski and Toth 2016; Van Regenmortel 1996). Synthetic peptide vaccines are preferred (Slingluff Jr 2011; Yang and Kim 2015) over the classical, live attenuated and subunit vaccines because of their characteristics like specificity, diversity, absence of allergic reactions to the pathogen and lack of cross immunization (Larché 2007; Mocellin et al. 2009; Slingluff Jr 2011; Yang and Kim 2015). The efficacy of peptide vaccines is influenced by several critical factors like physicochemical chattels of peptides (Lloyd-Williams et al. 1997; Smolenski et al. 1990; Tregear et al. 1973), excipients (Bjelošević et al. 2020) and process parameters (Connolly et al. 2015) involved in their formulation and development. Peptide vaccines are more stable in their secondary structure (Smolenski et al. 1990; Wiesmüller et al. 1989) than primary structure. Any alteration or shift in these stable forms generates denatured (inactive) peptide (Swain et al. 2013). Preservation of stable structures is an important challenge faced by immunologists (Buteau et al. 2002; Li et al. 2014). Peptide vaccine is usually lyophilized to yield a dry powder which consequently imparts stability to the sequence and protects it from external confounders (Connolly et al. 2015; Rexroad et al. 2002). However, lyophilization and other process parameters impart stress to the peptide moiety which may hamper the bioactivity of the product (Izutsu 2018).

Cryoprotectants are excipients that are incorporated in optimized manner to protect the peptide from stress inducing factors and providing stability (Kamerzell et al. 2011; Ohtake et al. 2011). Further, cryoprotectant shields the peptide molecular structure in a mechanistic pathway. Hence, selection of suitable cryoprotectant is crucial step in peptide vaccine product development (Bjelošević et al. 2020). For instance, 2% trehalose is added directly to Engerix-B vaccine to impart stability. Till date there are no marketed preparations of synthetic peptide vaccines (Skwarczynski and Toth 2016) although numerous synthetic peptide vaccines are being investigated in clinical trials (Table 1).

Therefore, in present review, the article enlightens the molecular mechanism of cryoprotectant in imparting stability to the peptide molecule. Furthermore, challenges associated with customization of synthetic peptide vaccines and the role of molecular dynamic studies and computational modelling in selection of a appropriate cryoprotectant in formulation and development of a stable and cost-effective synthetic peptide vaccines are also discussed.

Peptide Vaccine: What You Need to Know?

Vaccines are usually tailored using a dead or live attenuated strain of microorganisms that contain either the whole or part of the microorganism to elicit a specific immune response. Traditional vaccines have ruled over the global immunology market in twentieth century; however, conventional vaccines have their own pros and cons. Conventional vaccines are associated with autoimmune reactions or allergic shocks. Attenuated pathogen may reframe its virulent stage to cause life threatening hazards. In 1930s, the world experienced one of the worst cases in medical history owing to contamination in the vaccines. This disaster was later named as "Lübeck disaster". In this crisis, 67 neonates out of 249 were died post vaccination with Bacillus Calmette–Guerin (BCG) vaccine owing to contamination with virulent strains of *Mycobacterium tuberculosis* (Fox et al. 2016). All such incidents prompts humans to search for a safer, specific and more reliable version of vaccines called as synthetic peptide vaccines (Corran and Griffiths 1999). Synthetic peptide vaccines have exhibited several lucrative advantages over the conventional vaccines besides few limitations (Fig. 1). Synthetic peptide vaccines are laboratory products fabricated by fusing

Table 1 Clinical trials of synthetic peptide vaccines

Sr. no.	Synthetic peptide vaccines	Disease	Status
1	P53-Single long peptide vaccines	Ovarian cancer	Phase 2 NCT00844506
2	CML (chronic myelogenous leukemia) synthetic peptide vaccines	Chronic myeloid leukemia	Phase 2 NCT00267085
3	UV1 synthetic peptide vaccines	Non-small cell lung cancer	Phase 2 NCT01789099
4	UV1 synthetic peptide vaccines	Prostate cancer	Phase 2 NCT01784913

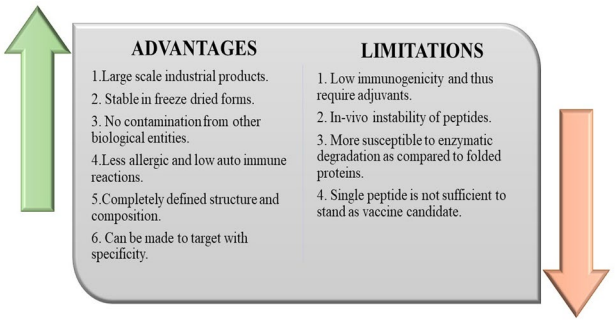

Fig. 1 Synthetic peptide vaccines exhibited several lucrative advantages over the conventional vaccines besides few limitations like low immunogenicity and instability

amino acids together to give a secondary structure of roughly 20–30 amino acids. The peptide sequence is very specific in its action and perfectly mimics the antigenic epitopes present on the microbes (Van Regenmortel 1996). Epitopes are antigenic determinants that are recognised by specific antibodies, B-cells and T-cells. These epitopes are the specific amino acid sequences that induce the immunogenic response. Recently, peptides engineered to self-assemble into specific nanoarchitectures have shown great potential as advanced biomaterials for vaccine development (Eskandari et al. 2017). Specifically, self-assembled peptides can provide cell adhesion sites, epitope recognition, and antigen presentation, depending on their biochemical and structural characteristics (Abudula et al. 2020).

Peptides are first recognized by the pattern recognition receptors (PPRs, Toll like receptors; TLRs) in order to elicit the immune response. Peptide molecules are then identified by the special type of cells called as antigen presenting cells (APCs) (For example: dendritic cells, DCs or macrophages) which subsequently engulf them. Further they undergo processing and are then presented to the major histocompatibility complex (MHC) proteins. These loaded MHC-2 proteins trigger the T-helper or CD4+ cells and lead to activation of cellular immunity or humoral immunity whereas, if the peptides are presented to MHC-1 proteins then it may lead to induction of cellular immunity via activation of CD8+ cells or T-cytotoxic cells (Fig. 2). This leads to production of antibodies in body that help in tackling the further ingress of antigens (Hos et al. 2018; Li et al. 2014; Skwarczynski and Toth 2016).

Antigen can be recognized, processed and transported to lymph nodes by peripheral APCs, or it may travel on its own to lymphatic nodes and then be processed by lymph node resident APCs. Lymph nodes are composed mostly of T-cells, B-cells, DCs and macrophages, and one of the major sites for activation of adaptive immunity (Itano and Jenkins 2003).

One of the vital features of immune responses is the T-helper subtype activation and corresponding type-specific cytokines release. Antigen loaded on MHC-II can activate both T_H1 and T_H2-types helper cells. T_H2 cells trigger mainly humoral responses against extracellular pathogens, while T_H1 cells activate cellular immunity against intracellular pathogen (viruses, cancer). However, T_H1 and T_H2 are not strictly equal with cell mediated and humoral immunity, respectively (Spellberg and Edwards Jr 2001). For example, the T_H1 pathway may also stimulate modest levels of antibody-based responses. T_H1 cytokines tend to produce the pro-inflammatory responses while T_H2 is associated with the anti-inflammatory responses. Imbalanced T_H1/T_H2 responses may cause immunopathological complications such as tissue damage via extensive inflammation or strong allergic responses (Alosaimi et al. 2020). Thus, a properly balanced T_H1 and T_H2 responses should be taken into account during the vaccine development process.

Synthetic peptide vaccines are produced in a very precise, orderly and step wise manner. The synthetic peptides are synthesized in the C-N manner, which means the C-terminal of the incoming amino acid is attached to the N-terminal of the growing chain. Exactly opposite process occurs during bio-synthesis of peptides. Synthetic vaccine produced, precisely and completely mimics the antigenic epitope. The first step in synthetic vaccine production involves complete study of the antigenic epitopes. This is followed by sequencing of amino acids to render the actual required peptide sequence. They are normally synthesized either in the solution form or in the solid form (Merrifield 1969). Production of peptides becomes simple, easily reproducible, fast and cost-effective due to recent developments in solid phase peptide synthesis (SPPS) using automatic synthesizers and application of microwave techniques (Skwarczynski and Toth 2016). Chemical synthesis practically removes all the problems associated with the biological contamination of the antigens. It is well known that amino acids have a large number of active functional groups present in their backbone which can hamper the process of peptide synthesis. Thus, blocking or protecting groups are used to avoid these reactions. Techniques like TASPs (Template Assembled Synthetic Proteins) or MAPs (Multiple Antigenic Peptides) are used to provide the backbone for the easy and systematic synthesis of viable and functional peptides (Corran and Griffiths 1999). Peptides can be customized to target very specific objectives. The immune responses can be directed against naturally non-immunodominant epitopes. By the use of a multiepitope approach, single peptide-based vaccine can be designed to target several strains, different stages of life cycle or even different pathogens.

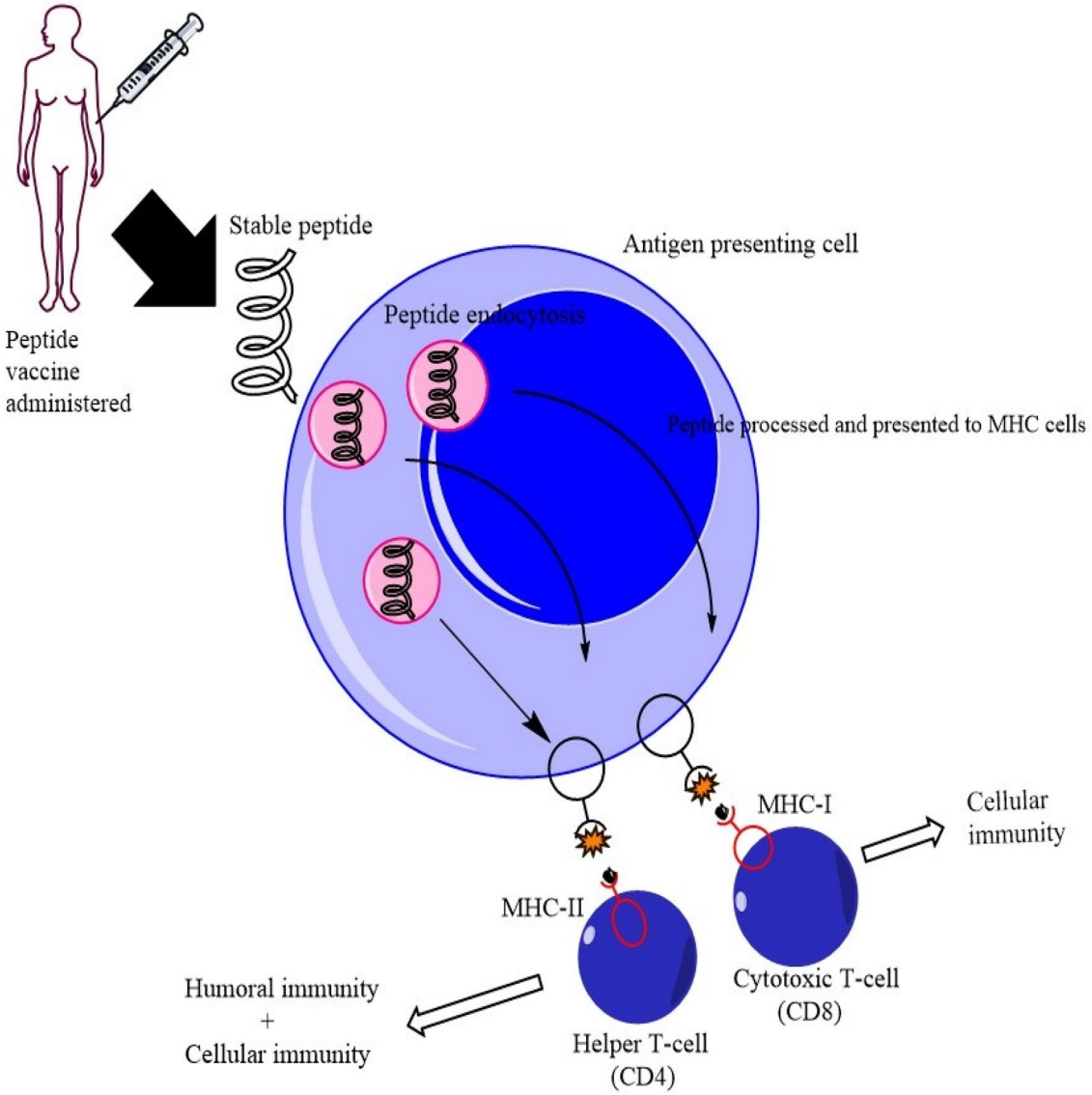

Fig. 2 Peptides are recognized by the pattern recognition receptors (PPRs, Toll like receptors; TLRs) in order to elicit the immune response. Peptide molecules are then identified by antigen presenting cells (APCs). Further, they presented to the major histocompatibility complex (MHC) proteins and trigger the T-helper or CD4+ cells and lead to activation of cellular immunity or humoral immunity

Physicochemical Limitations of Synthetic Peptide Vaccine: Does Stability of Peptide Influences Efficacy?

There are many variables that need to be considered during the synthesis of an active peptide molecule as they govern the stability, potency, viability and efficacy of the peptide molecules synthesized (Fig. 3).

Solubility

All peptides are manufactured primarily in the aqueous solutions then they are dried so as to maintain their stability during storage and are again reconstituted in suitable media. Because of these parameters it is very important to modulate the solubility of the peptide molecule being synthesized in a desired manner. The solubility of the peptide molecule is

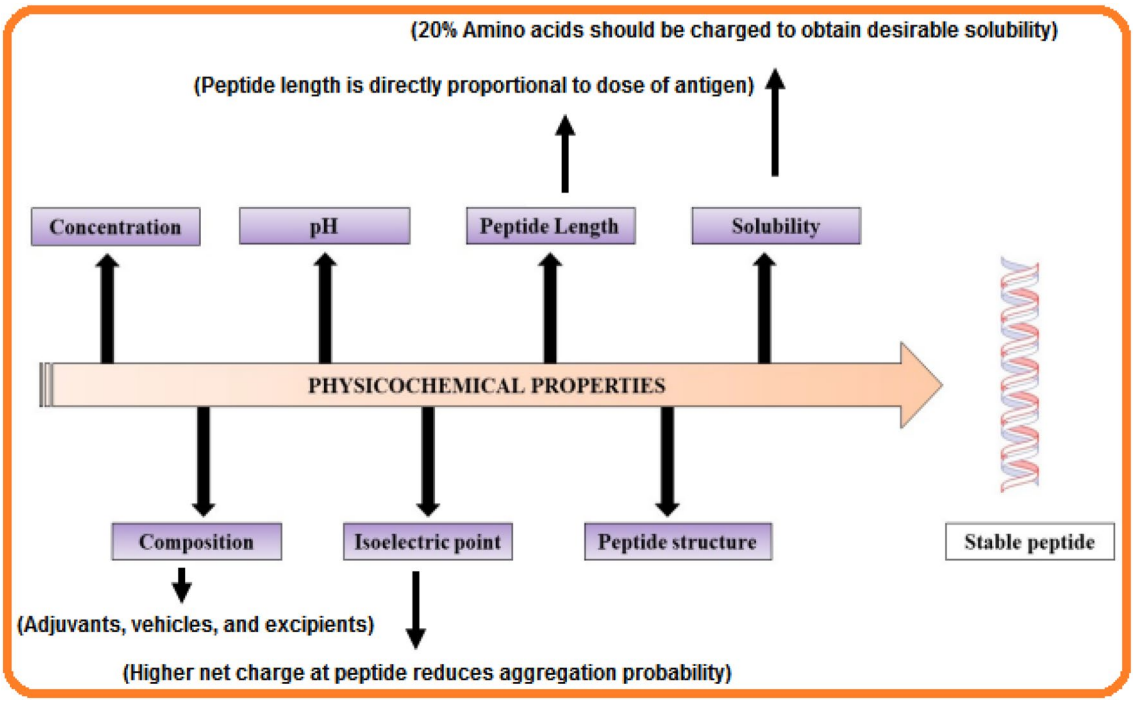

Fig. 3 Physicochemical properties like solubility, pH, isoelectric point influence the stability of peptide. For example, 1 out of 5 amino acids should be charged to obtain sufficient solubility in aqueous medium. On the other hand, at pH below isoelectric point, the net charge is positive and above isoelectric point, it is negative. Any alteration in pH equivalent to the isoelectric point drastically change the physicochemical properties

governed by the amino acids from which it is constructed. Amino acids are classified into hydrophilic, hydrophobic, acidic, basic and amphiphilic according to their structures (Satyanarayana and Chakrapani 2013).

The proportion of polar and nonpolar amino acids in the sequence will dictate the solubility. As a thumb rule it is considered that 1 out of 5 amino acids should be charged to obtain sufficient solubility in aqueous medium. Thus, to maintain this, structural amino acids can be replaced as per the requirement (Satyanarayana and Chakrapani 2013).

Concentration of Peptides

Concentration of the peptide in synthetic peptide vaccine formulation depends on dose which in turn calculates the immunologic response, stability and toxicity of the peptide. Concentration of the peptide also depends on their physical stability as well. The physical stability of the synthetic peptide vaccine decreases as a function of the concentration of the peptides which consequently undergo aggregation phenomena (Wei et al. 2017). The peptides first form oligomers and then the nucleus goes on increasing to form huge aggregates (Zapadka et al. 2017). It is also found to be true that if the concentration of peptides is kept high then the loss of peptides that takes place during freezing and drying will not hamper the actual active concentration in the synthetic peptide vaccine formulation (Wei et al. 2017). Thus, there are many contradictory conclusions drawn towards the actual concentration of peptides.

Net Charge on Peptides

Isoelectric point is the pH at which the peptide molecule effectively has no net charge. At isoelectric point there is a total abstinence of electrostatic repulsive forces because of which there is increased aggregation of the peptides (Novák and Havlíček 2016). This explains the loss of solubility of the peptide at isoelectric point. The pH of a solution will determine the net charge on the surface. Thus, the pH of the final solution has to be architecture very carefully. In general, if the net charge is higher, then the chances of aggregation are lowered (Jain and Udgaonkar 2010; Kamihira et al. 2003; Klement et al. 2007; Marshall et al. 2011; Raman et al. 2005; Yun et al. 2007; Zapadka et al. 2017). At pH below isoelectric point the net charge is positive and above isoelectric point it is negative. The pH of the solution is maintained by inclusion of buffers. During the processing of the peptides, excipients tend to crystallize out and thus lead to drastic change in the pH of the solution. If the pH gets changed to anywhere near the isoelectric point then its

properties will change drastically. This makes it mandatory to carry out tests for stability of the product before marketing it.

Adjuvants, Vehicles and Excipients in Synthetic Peptide Vaccines

A wide range of different materials can theoretically be combined into a synthetic peptide (immunogen) in order to improve handling, stability, and persistence and to modify the degree and type of immunogenicity. Such materials vary widely in their characteristics and range from small molecules to bacterial cell components and synthetic polymers. They may include components acting as general immunostimulants, components selectively activating different parts of the immune system and components interacting directly with the different cells involved in the immune process. Many adjuvants may present questions of toxicity, and the way in which they modify the immune process raises safety concerns when combined with an antigen.

Established adjuvants based on aluminium or calcium salts should be of the requisite pharmacopoeial grade (i.e. free of heavy metal ions and of consistent quality and binding characteristics). Since operations such as autoclaving may alter an adjuvant's binding characteristics, it is important that batches of adjuvant should be handled in the same well-defined manner. If aluminium or calcium compounds are used as adjuvants, their concentrations should not exceed the customary limits of 1.25 mg of aluminium and 1.3 mg of calcium per single human dose.

Excipients in peptide formulations need very careful evaluation and selection because of the inherent sensitivity exhibited by peptides themselves (Bjelošević et al. 2020). Peptide formulations are finally stored in freeze dried state so the formulators must consider this while selecting their excipients because not all excipients are stable under such situations of stress. Excipients normally used in peptide formulations are stated in Table 2 with the role they play. All the selected excipients have one basic aim to protect the native structure of the peptide molecules and to protect them from the stresses generated during the production and storage of the final formulation (Kamerzell et al. 2011; Swain et al. 2013). Adjuvants are the excipients of specific importance when peptide vaccines are considered. Peptide vaccines by virtue of their nature have been proved to have low immunogenic properties. This is regarded to their small size and varying availability of APCs at the site of administration. Thus, peptide molecules are always administered with adjuvants which function to improve the immunogenic response generated. They help in presenting the peptide molecules to the APCs and contribute towards enhancing the immunogenic response. In the earlier days alum was used as an adjuvant but now newer and safer adjuvants like Freund's adjuvant have been reported to be used on a large scale. All the other excipients (Table 2) act as stabilizers and increase the potency of peptide molecules in one way or the other.

Synthetic peptide vaccines have potential to generate a holistic change in the field of vaccination but they have faced plethora of challenges associated with their development from lab scale to commercial market which are listed in Fig. 4.

Cryoprotectant: A Shield for Peptide Vaccines

Peptide molecules are very sensitive in nature and easily undergo degradation in solution forms thus they are converted into solid state where they are considerably more stable. Peptides and proteins normally undergo spray drying or lyophilization processes to convert them from solution form to solid state. Spray drying has been exploited more in food industry and lyophilization in pharmaceutical industry. Lyophilization aims at removing water from frozen products by the process of sublimation. It involves three basic steps-freezing, primary drying and secondary drying. In the first step the temperature of the formulation is drastically reduced which leads to formation of ice crystals that cause severe damage to the sensitive structure of peptides. During the

Table 2 Excipients used in peptide vaccines

Excipients	Role of excipients	Examples
Adjuvants	Enhances immunogenic response	Aluminium salts, Freund's adjuvant
Cryoprotectants	Maintain viable structure of peptide during lyophilization	Trehalose, proline, sucrose, etc
Buffers	Stabilize pH change in the medium	Phosphate and carboxylate buffers
Osmolytes	Stabilizing cosolvents	Sugars, polyols, etc
Amino acids	Stabilizers, buffers and antioxidants	Arginine, histidine
Salts	Tonicifying agents (follows Hofmeister series)	Sodium chloride (NaCl)
Surfactants	Surfactants prevent aggregation of peptide and reduce surface tension	Non-ionic surfactants, polysorbate 20, polysorbate 80

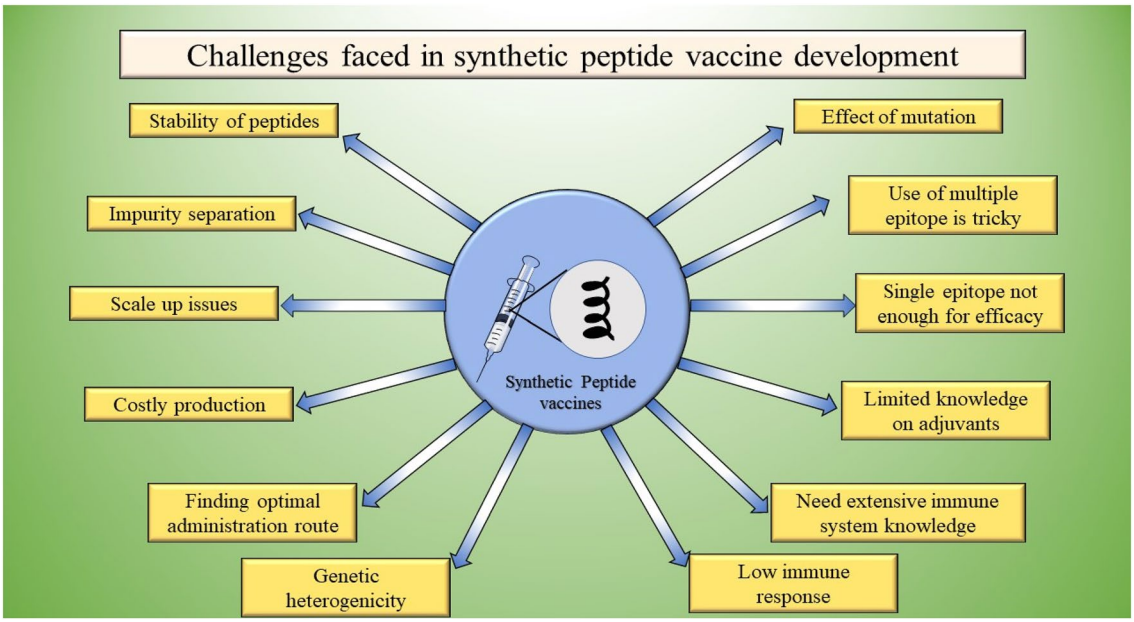

Fig. 4 Summary of the challenges faced in synthetic peptide vaccine development like scale-up issue, high production cost, low immune response, stability of peptides etc.

consecutive step the temperature is raised under pressure to evaporate water by the process of sublimation and render the product dry. The final drying step also called as desorption is performed to eliminate any traces of bound water in the formulation (Bjelošević et al. 2020; Izutsu 2018; Rexroad et al. 2002). The amount of moisture to be retained in the final formulation is totally dependent on the inherent properties of the formulation and is obtained by optimization process. During lyophilization process peptides face stresses like freezing, drying and solid–liquid interfacial tension. The interfacial tension problem between solids and liquids is resolved by inclusion of surfactants in the formulation. But to combat the problems of freezing and drying stress, excipients called as cryoprotects and lyoprotectants respectively are incorporated. Here in peptide vaccines, this means protection from ice formation during the lyophilization process. Cryoprotectants have been broadly classified into two types- penetrating and non-penetrating agents. Penetrating cryoprotectants have been studied to protect the cells during dehydration conditions, they are generally osmotically inactive (Sieme et al. 2016). Dimethyl sulfoxide (DMSO) forms the pivotal example of penetrating cryoprotectants. The non-penetrating cryoprotectants are either sugars like trehalose and sucrose (Fig. 5) which are osmotically active or are polysaccharides, like starch and dextran, and proteins which are osmotically inactive compounds (Sieme et al. 2016). All these agents have been extensively studied and worked upon for their mechanism of action, their optimum concentration and their toxicity profile which will be talked upon shortly.

Cryoprotectants-Mechanistic Insight

Degradation leads to loss in functionality of the peptides (Swain et al. 2013). There are two broad types of degradative mechanisms faced by peptides—physical and chemical degradation. Chemical degradation includes reactions like oxidation, de-amidation, maillard browning and moisture sensitivity (Mensink et al. 2017). Stability of peptides can be enhanced by proper packaging and by turning the peptides into solid formulation (Bjelošević et al. 2020). Physical degradation includes denaturation and non-covalent aggregation. Denaturation leads to unfolding of the structure of the peptides and thus leads to exposure of the hydrophobic groups, which were protected to the aqueous surroundings and leads to loss of activity (Lai and Topp 1999). Non-covalent aggregation can be reduced by use of stabilizers and cryoprotectants. Various theories are postulated to discuss and define the mechanism of actions of cryoprotectants. DMSO was most widely used agent as a cryoprotectant and it acts by forming aqua pores in the lipid bilayers and thus regulating the effect of dehydration on the particular cell. But DMSO has found no use in the field of synthetic peptides because they lack the phospholipid bilayer around them. But if peptides are formulated inside any nanoparticles like liposomes then DMSO can be exploited but still its use will be rationed by its toxicity profile. Most widely accepted cryoprotectant agents for synthetic peptides include sugars, saccharides, few polymers and proteins.

Widely accepted theories for mechanism of action of sugars are (1) vitrification theory, (2) water replacement theory

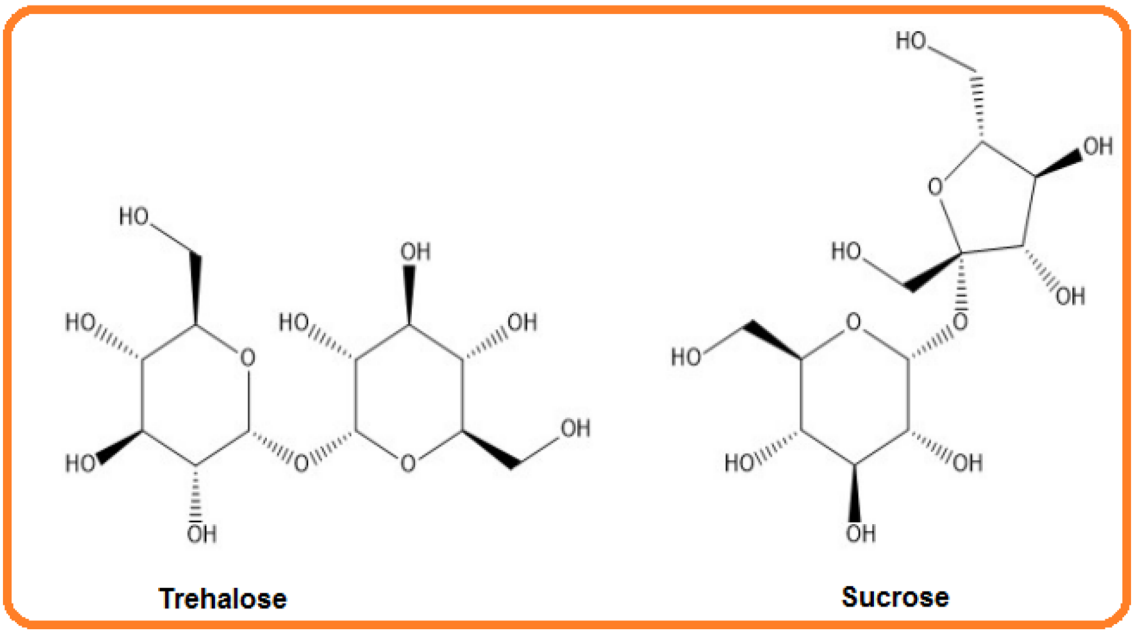

Fig. 5 Chemical structures of commonly used cryoprotectants lie trehalose and sucrose

and (3) water sequestration theory (Izutsu 2018; Sieme et al. 2016) (Fig. 6). Vitrification theory speaks about formation of a glassy, sugary coating around the peptide molecule which reduces the overall mobility of the peptides and increases its stability. Because of the decrease in mobility of the peptides it provides kinetic stability to them. Larger saccharides like oligo saccharides and polysaccharides are believed to act by this mechanism. Here there is no direct bond formation between the cryoprotectants and the peptide molecules. Glass transition temperature (T_g) is the temperature where the product converts from viscous nature to more fluidic nature. Every sugar molecule has a specific glass transition

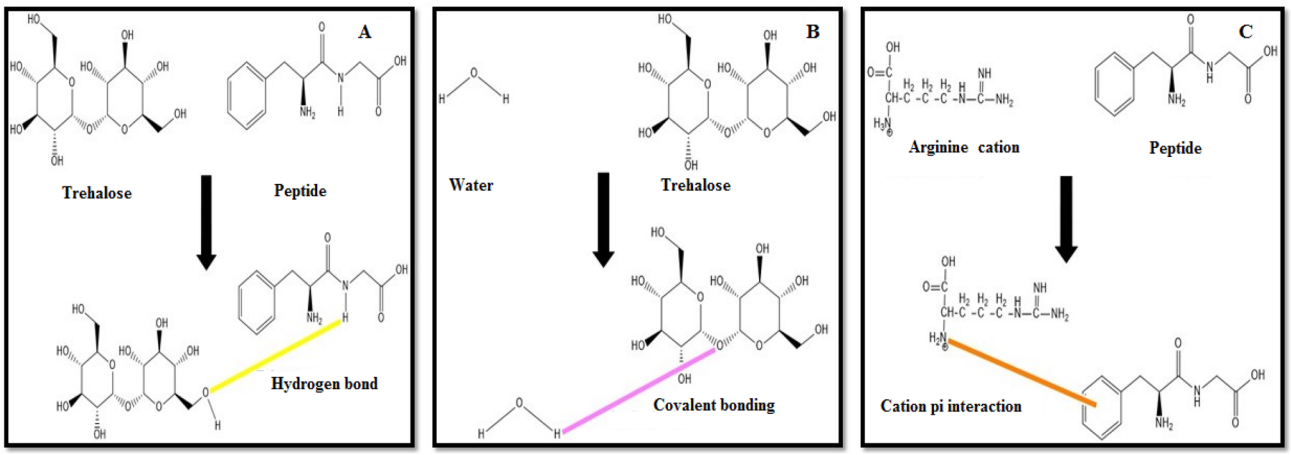

Fig. 6 Mechanism of action of cryoprotectants under **a** Water replacement theory. Small sugar molecules like trehalose possess better flexibility than larger and rigid sugars. If the sugars have a greater number of hydrogen bond forming groups and are small in size then they can easily enter the grooves of the peptide molecules and can decrease the free volume by filling all the voids. **b** Water substitution theory. The oxygen molecules of trehalose from their glycosidic bonds interact with the surrounding water molecules and thus structure them around trehalose rather than around the peptide molecules. This leads to reduction in the stress caused due to dehydration of the preparation. It effectively reduces the amount of water available around peptides for freezing and thus reduces the freeze and drying induced stress. **c** Cation-pi interaction theory of amino acids. Arginine increases the stability of proteins through electrostatic interactions and also through cation-pi type of interactions. Arginine interacts with the hydrophobic portions of the proteins (through its $–CH_2$ groups) and augments the stability

temperature above which it loses its activity as a cryoprotective agent. The effect of T_g of every agent is dictated by the storage temperature of the product. The effect of T_g on different formulations which were freeze dried using lyophilization technique was studied extensively (Sieme et al. 2016). If the difference between the storage temperature and T_g of the cryoprotectant is very less than the vitreous theory is exemplified. During such circumstances the cryoprotectant converts itself into a glassy state because of which the ultimate viscosity of the medium is increased. This resultant viscosity leads to decrease in the free movement and mobility of the peptide molecules and thus reduces its interaction with the environment as well as inhibits its unfolding and eventually protects it from getting de-stabilized. The immobilization established by vitrification can be described as global (alpha relaxation) but inside the peptide structure there is still some local mobility (beta relaxation) which is the major reason for chemical degradation faced by peptides (Mensink et al. 2017). In a study it was found that the glassy matrix is impermeable to oxygen and thus proves to be useful in avoiding chemical reactions to some extent (Tromp et al. 1997; Ubbink and Krüger 2006). The stability of proteins can thus be correlated to their local mobility inside this inert matrix provided by cryoprotectants.

Second theory is the water replacement theory. This theory actually involves bond formation between the peptide molecule and the cryoprotectant. The peptide molecules are synthesized using amino acids, thus there are many groups which form hydrogen bonding with the water in the environmental fluid. When lyophilization process is performed on the peptides the water present in the medium is converted into ice and then sublimated. These hydrogen bonds formed between peptides and water molecules causes the peptides to undergo degradation. Cryoprotectants tend to substitute water molecules from the surface of peptide and thus form hydrogen bonds with peptide molecule. These bonds formed provide stability to the peptides and reduce the entropy of the system. Thus, they provide thermodynamic stability to the system. They actually substitute the thermodynamic instability that peptides undergo during drying process. The cryoprotectants maintain the native structure of the peptides by bonding with them (Chang et al. 2005). Jain and Roy (2009) have studied this theory for trehalose by using Fourier transform infrared (FTIR) technique. FTIR is a very sensitive method that is used to identify the presence and absence of functional groups in a compound. The binding of cryoprotectant with peptides maintains the native structure and bonds observed in the peptide. Carpenter and Crowe (1989; Mensink et al. 2017) studied these interactions (–OH groups of sugar bonds with –NH groups of the peptide and stabilize it) and concluded that the formation of hydrogen bonds provide stability to the peptide molecules. It has been observed that small sugar molecules that possess better flexibility act by this mechanism rather than larger and rigid sugars. If the sugars have a greater number of hydrogen bond forming groups and are small in size then they can easily enter the grooves of the peptide molecules and can decrease the free volume by filling all the voids (Mensink et al. 2017). As discussed earlier the T_g of sugars play a key role in defining the efficacy of cryoprotectants. Thus, for sugars to act by water replacement theory the difference between the storage temperature of the product and the T_g of the cryoprotectant should be 10–20 °C. Few studies also revealed that sugars interact with the peptide molecules through a CH-pi interaction, where the sugars were found to bond with the aromatic rings present in the peptide amino acids. The CH-pi interactions are actually more pronounced during binding of peptides to their active sites or ligands. The –CH groups attain a slight acidic nature and the aromatic rings gain the positive charge. This has been studied extensively in many reports (Kamerzell et al. 2011; Laughrey et al. 2008; Stanca-Kaposta et al. 2007).

Most of the known cryoprotectants exhibit their protective action by above two mentioned mechanisms. No cryoprotectant can work exclusively by one mechanism and provide complete protection to the peptide molecules. They can work using both the mechanisms, but the fact that which mechanism will predominate will be determined by the working and storage conditions of the final product and the T_g of the cryoprotective agents.

The third mechanism is exhibited by very few agents and has been studied exclusively and majorly on trehalose (Novák and Havlíček 2016). Trehalose was found to order the structure of water molecules by specifically separating them from the peptide chunk of the preparation. Thus, trehalose like molecules are termed as kosmotropes. These agents are shown to exhibit water structuring properties and thus increase the order of water molecules. This effectively is attributed to the fact that the strength of covalent bond between water molecules is less as compared to the strength of covalent bond between trehalose and water molecules. The oxygen molecules of trehalose from their glycosidic bonds are shown to interact with the surrounding water molecules and thus structure them around trehalose rather than around the peptide molecules. This leads to reduction in the stress caused due to dehydration of the preparation. This mechanism is also famous by the name of "solute exclusion" where water exclusively binds with trehalose or "water entrapment" (Belton and Gil 1994; Sieme et al. 2016). It effectively reduces the amount of water available around the peptides for freezing and thus reduces the freezing and drying induced stress (Cho et al. 1997; Liu and Brady 1996; Novák and Havlíček 2016).

The distinct type of cryopreservatives which include agents like polysaccharides and proteins are added into the formulation as they work on different levels and perform

different functions at the same time. They have been found useful as antioxidants, stabilizers, buffering agents and are even effective as cryoprotective agents. Few investigators have concluded that proteins like proline, arginine, histidine, etc. act through the mechanism of water replacement. But others confirm that proteins act through hydrophobic interactions and present their protective action. Trout et al. have done extensive studies on the mechanism of action exhibited by arginine as a cryoprotective agent. They confirmed that arginine increases the stability of proteins through electrostatic interactions and also through cation-pi type of interactions. It forms bonds with the charged moieties on the amino acids in the peptide to form electrostatic interactions and with the aromatic residues to form cation-pi interactions. In their further works they found that arginine interacts with the hydrophobic portions of the proteins (through its methylene groups) along with the aforementioned mechanisms and thus provides enhanced stability to the preparation (Baynes et al. 2005; Kamerzell et al. 2011; Shukla and Trout 2010, 2011). In this manner arginine and similar amino acids can be used to enhance the stability of peptides by inhibiting their aggregation. Polymers like dextran have also been studied for their ability to act as cryoprotectants. The ability of the polymers to act as cryoprotective agents depend on their intrinsic nature. Hydrophobic polymers, unlike proteins, destabilize peptides by enhancing their aggregation. Hydrophilic polymers have been proven to increase the stability. Polymeric materials can also act through electrostatic interactions with the charged moieties on the peptide surface (Azevedo et al. 2004; Ohtake et al. 2011; Shtilerman et al. 2002; Uversky et al. 2002; Zhang et al. 2007).

Selection Criteria of Cryoprotectants and Their Implications in Synthetic Peptide Vaccines Development

Effect on T_g

One of the parameters that have impact on selection of cryoprotectant for peptide vaccine includes T_g. T_g can be viewed as the temperature at which the viscous nature of the molecule turns into fluid like state. T_g is the characteristic temperature and is unique for every material or polymer (Jain and Roy 2009). Materials which are well below T_g exhibits much slower structural relaxation time and reduced mobility (Simperler et al. 2006).

T_g determination is one of the important criteria while selecting cryoprotectant. It indicates the level of stability that will be generated in the formulation. If the difference between storage temperature and glass transition temperature accounts for (10–20 °C), it suggests that water replacement theory plays a predominant role. If the glass transition temperature and storage temperature lie in proximity of each other, it suggests that vitrification theory acts as a limiting factor (Mensink et al. 2017). Difference between the storage temperature and T_g should be optimum so as to ensure the stability of peptides in vaccine systems. If the T_g of the selected cryoprotectant is lower and closer to storage temperature, it will lead to increase in molecular mobility among peptide molecule as the conversion from viscous to fluidic state is faster. Faster conversion will ultimately result into faster inactivation of peptides owing to its mobility in the system. In order to retain the therapeutic efficacy of peptide-based vaccine, selected cryoprotectant should possesses higher T_g but, T_g alone is not the only guiding factor governing therapeutic efficacy (Mensink et al. 2017).

The T_g for glucose, sucrose and trehalose were determined experimentally and followed a trend like pattern of T_g glucose (296 K) < T_g sucrose (333 K) < T_g trehalose (380 K). Hence, trehalose can act as an effective cryoprotectant as it possesses higher T_g value than sucrose thus imparting stability to peptide molecule (Simperler et al. 2006). Polymers such as dextran also increases the T_g of material (Kamerzell et al. 2011).

Addition of other excipients like salts, buffers and presence of moisture causes deleterious effects by lowering the T_g of the material and in turn hampering the stability of the molecule (Simperler et al. 2006). T_g also plays an important role by dictating the mechanism by which the cryoprotectant is likely to act. T_g of the material is conventionally calculated by DSC thermograms. Advance techniques including molecular simulation (in silico) methods are used to estimate T_g values. It is found that molecular dynamics, which is based on free volume theory, overestimates the T_g value than the actual value but essentially portrays the same trend as seen experimentally. The method employed in this technique consists of graphical plots of increasing and decreasing temperature branches. The intersection of those branches provides T_g value at which the glassy state of material is converted to rubbery state (Simperler et al. 2006).

In an experiment to determine the effects of Trehalose on monoclonal antibody (mAb) formulations, samples were cooled fast and were stored for 12-months study at − 20 °C and − 40 °C temperature. It was found that samples stored at − 20 °C showed presence of crystals and protein denaturation but the samples which were stored at − 40 °C showed no presence of crystals and also the native structure of protein was preserved. Higher degree of stability seen in samples stored at temperature − 40 °C is due to the fact that the storage temperature was well below the glass transition temperature and thus helped in safe guarding the protein native structure (Connolly et al. 2015).

Effect on Peptide Conformation and Stability

In order to maintain the therapeutic efficacy of the drug delivery system, peptide stability is of utmost importance. Loss in the stability of peptide molecule leads to inactive peptide an in turn resulting in failure to exhibit therapeutic response. Peptide molecules exist in a stable conformation in the system by the virtue of folding. Peptides which are composed of protein units, expresses negative adsorption isotherms on the protein surface (Randolph 1997). Every system in universe tries to attain minimum surface free energy. Proteins and peptides achieve minimum surface free energy by existing in most compact form which involves structural folding without disturbing the stability.

When peptide vaccines are formulated, peptide molecules undergo various stresses such as freezing, dehydration, pH changes or increase in ionic strengths (Bjelošević et al. 2020). These stresses cause surface-induced changes in a peptide molecule and may result in destruction of stable structures or may induce thermodynamic effects due to temperature changes (Randolph 1997). During freezing cycles, over drying produces major hurdles in terms of stability if system is devoid of optimum residual water (Roy and Gupta 2004). Peptides are sensitive to change in temperature, pH, pressure, etc. Cryoprotectants are added in order to preserve this native structure of peptide molecules (Arakawa et al. 1993).

Cryoprotectants like trehalose act by vitrification theory or via water replacement theory by replacing the bonds between polar groups of protein and water with cryoprotectant itself. Cryoprotectant which is most excluded from protein surface is termed as the best. Cryoprotectants must be in amorphous state so as to stabilise the peptide molecules (Izutsu et al. 1993; Jain and Roy 2009).

When a protein structure unfolds there is an increase in exposed surface area of molecule (Randolph 1997). Cryoprotectant like trehalose inhibits this unfolding of protein molecule and favors the maintenance of native chemical structure thus restoring chemical potential. Correlation between protein mobility in glass matrix and T_g corresponds to stability of protein molecule. Glassy transition state exerted by trehalose leads to complete inhibition of mobility of protein molecule whereas bond formation mechanism on the other hand prevents the interaction of protein with external water molecule thus guarding the protein structure (Bjelošević et al. 2020).

Addition of excipients like buffers, salts, bulking agents also contribute to loss in protein stability. Buffer crystallises during freezing cycle and thus lowering pH (nearly 3.6–4) which destabilises the molecule. Bulking agents may phase separate thus presenting challenge in maintaining stability of protein molecules (Randolph 1997).

Low molecular weight disaccharides (like sucrose) are often the choice of excipient as they are excluded from protein surfaces by the virtue of localisation at protein water interface than being present in polymer system. Also, relaxational kinetics of low molecular weight disaccharides are much lower than large molecules thus reducing mobility (Randolph 1997).

Protein aggregation is a form of instability of the system and is related to sugar size (cryoprotectant) and its miscibility. In order to prevent aggregation of protein molecule, low sugar size along with higher sugar miscibility is preferred (Mensink et al. 2017).

Mannitol which is a cryoprotectant has a good effect on stabilising proteins during freeze drying but it is least efficacious in its crystalline state and is not a commonly used cryoprotectant. Sorbitol also possesses no detrimental effects on protein stability but has tendency to crystallise as it has characteristic low T_g. Inositol expresses concentration dependent effect on protein stability (Bjelošević et al. 2020; Izutsu et al. 1993). β-galactosidase when freeze dried with trehalose and sucrose as cryoprotectants showed no loss in activity during cycles of freeze drying owing to decrease in mobility due to glassy matrix formation whereas glucose and fructose were ineffective (Ohtake et al. 2011). Several sugars (sucrose, lactose, maltose) exhibited protective effect on poly L-lysine by preventing its random coiling transition occurring in beta sheets (Kamerzell et al. 2011).

Various polymers like dextran, diethylamino ethyl (DEAE) help in preserving the protein stability as was observed with lyophilised bovine serum albumin (BSA) samples where protein aggregation was found to be decreased (Kamerzell et al. 2011). Polymers can bind to specific sites on proteins and thus stabilise their quaternary structure via preferential exclusion (Ohtake et al. 2011).

Optimization of Concentration of Cryoprotectants for Peptide Vaccines

Concentration of cryoprotectant plays a chief role in deciding the stability of the formulation. Change in concentration of cryoprotectants and proteins both impact the fate of the system. Cleland et al., carried out a study to determine the effects of different molar ratios of sugars on protein formulations and demonstrated that the sugar (trehalose) and protein (recombinant humanised mAb human epidermal growth factor receptor 2 (HER2)) existing in ratios of 360:1 respectively provided optimal stabilisation and protective effect (Cleland et al. 2001). Any increase in the ratio of sugar leads to depression in T_g value and thus causing stability related issues in the system (Bjelošević et al. 2020). If the trehalose concentration is increased beyond optimum concentration it leads to increase in protein aggregation (Kamerzell et al. 2011). Increase in concentration of cryoprotectants

may show either increase or decrease in the aggregation of protein molecules as it is affected by another factor which is rate of freezing (Lee et al. 2009).

A study was orchestrated in order to evaluate the effects of protein aggregation by varying the trehalose concentration. The samples were studied for duration of 12 months testing at temperatures of − 20 °C and − 40 °C to check the presence of crystallinity or protein aggregation within the formulation. Trehalose was used in working ranges of (0–34.2%w/v). It was found that as the concentration of trehalose increased beyond the optimal concentration, crystallinity and protein aggregation also increased which was studied by FT-NIR (Fourier transformed near infrared radiation spectroscopy) and SEC (size exclusion chromatography) techniques. If trehalose is used in lower concentration than the optimal range only protein aggregation was seen. Though the concentration of cryoprotectant is not the only criteria that governs the stability of system but also it depends on the T_g of the cryoprotectant (Connolly et al. 2015).

The effect of variable concentration of trehalose on lysozyme preparation was evaluated as it was found that, higher trehalose concentration induced inhomogeneity with presence of protein aggregation near the ice freeze concentrate interface in the system where ice and freeze concentrated liquid coexist. At lower concentration of trehalose near to optimal range resulted in conservation of native structure of molecule (Kamerzell et al. 2011). In a study it was found that increasing the concentration of trehalose resulted in decrease in activity of phosphofructokinase (Ohtake et al. 2011).

Effect on Freeze Cycle Time

During lyophilisation and freeze drying of peptide vaccines, one of the key process parameters that affect peptides and proteins is freeze cycle rate. Freezing stage induces many structural and physicochemical changes in peptide molecule (Connolly et al. 2015).

Freezing rate is directly proportional to the driving force of the system which is the difference in temperature between the cooling surface and liquid-ice front. It is difficult to measure the freezing rate but may well correlate with controlling the cooling rate (Connolly et al. 2015). It was found that keeping the cooling rate to fast mode (> 100 °C/min) leads to crystallisation of the cryoprotectant (mainly trehalose into trehalose dihydrate) and eventually affected the protein stability. Whereas operating on slow mode (< 1 °C/min) helped in preserving the amorphous nature of the cryoprotectant and also had no effect on protein stability (Connolly et al. 2015).

It was also found that slow cooling rates lead to better re-dispersibility of product as found with BSA/mAb samples when they were cooled on slow mode (0.5 °C/min) (Bjelošević et al. 2020; Lee et al. 2009). Faster cooling leads to the formation of numerous small ice crystals thus creating larger interfacial surface area thus proposing the chances of nucleation. Whereas operating on slow cooling mode resulted in larger ice crystals and lower propensity towards nucleation which can be easily controlled to avoid crystal formation (Connolly et al. 2015).

Protein aggregation is directly related to crystalline trehalose dihydate form rather than amorphous nature. It was found out from a study conducted by subjecting mAb samples with different trehalose concentrations to three modes of cooling process: fast, intermediate and slow with concentrations of trehalose as (2.1%w/v, 5.4%w/v, 8.2%w/v) which were stored for 12 months at − 20 °C temperature. Results analysed through FT-NIR, turbidimetric analysis and SEC generated data show that protein aggregation was mainly found to be maximum at 5.4%w/v and 8.2%w/v in rapidly cooled samples as compared to slow cooled samples which showed no protein aggregation due to lower ratios of trehalose dihydate present. Size, shape and directionality of ice crystals depends on cooling rate thus maintaining optimum cooling rate during the formulation of peptide vaccines is necessary to maintain its effectiveness (Connolly et al. 2015).

Effect on Toxicity

Best cryoprotectant is one which serves its role of protecting protein/peptide molecule with minimum toxicity. It is generally found that increase in the cryoprotectant concentration leads to increase in toxicity (Best 2015). Glycerol and DMSO act as penetrating cryoprotectant of which DMSO is said to be a gold standard for preserving biological substances but the problem encountered with the use of DMSO is its potential to induce toxicity (Fahy et al. 1990). The correlation between the toxicity of the cryoprotectant and the protein instability can be drawn frequently (Best 2015).

DMSO is found to oxidise the sulfhydryl groups on proteins and leads to oxidative damage (Snow et al. 1975). DMSO, glycerol, especially propylene glycol leads to the formation of potential toxic formaldehyde intermediate by non-enzymatic reactions (Best 2015).

According to protein denaturation hypothesis, strong cryoprotectant has ability to form strong bonds with surrounding water which also governs for the possibility of bond formation between cryoprotectant and protein surface which will lead to protein denaturation with colligative interference from ice front (Arakawa et al. 1990).

Whereas according to the dehydration damage hypothesis, strong cryoprotectant may act as toxic species by reacting strongly with hydration layer of water such that it devoids the peptide from minimum water required for its stability inducing dehydration damage (Arakawa et al. 1990).

Fahy et al., devised a method to quantify the toxicity of several cryoprotectant by a following formula:

$$qv * = \frac{\text{Moles of water}}{\text{Moles of polar groups on penetrating cryoprotectants}}$$

where v is the concentration needed to vitrify biomaterials under standard conditions (*) and qv* varies linearly with toxicity and glass forming ability.

Other cryoprotectants has values of qv* in range of 2–4 whereas DMSO has value of 6. From this it was concluded that lower qv* will result in lower toxicity (Best 2015). Due to these problems faced in penetrating cryoprotectants, researchers propose use of non-penetrating cryoprotectants that are efficient in action without producing toxicity (Best 2015). Trehalose which is widely used cryoprotectant in protein-based pharmaceuticals is a non-penetrating cryoprotectant having higher T_g, higher stability, non-toxicity and is recognized as GRAS (Generally regarded as safe) excipient by US-FDA in the year 2000 (Richards et al. 2002).

Is Combination of Cryoprotectants Advantageous or Not?

Using one or two cryoprotectants may offer synergistic effect depending upon the selection of categories of cryoprotectants. This selection should be made considering that in a combination, individual cryoprotectant may exert different characteristic changes in any of the parameters in processing of peptide vaccines.

As discussed earlier, T_g plays principal role in selection of cryoprotectant and also in inducing stability in systems. Combination of large polysaccharides with small disaccharides or oligo saccharides leads to increase in T_g of cryoprotectant (Mensink et al. 2017).

Using PEG as polymer along with dextran and sucrose tends to reduce the phase separation and ensure protein stability. Garzon-Rodriguez et al., studied the effects of combination of hydroxyethyl starch with sucrose or trehalose were shown to increase T_g as compared to the use of hydroxyethyl starch alone. Combination of simple disaccharides with polysaccharides were shown to depict positive results with protein stability (Bjelošević et al. 2020).

Amino acids are used as a buffering agent but also can act as stabilisers and different combination studies were reported. Glycine was found to show synergistic effect with disaccharides mainly with sucrose. Arginine which is one of the amino acids works on the principle of weak surface interaction and prevents binding interactions between proteins. Various amino acids were found to show interesting effects when they were combined with disaccharides or sugars. Forney-Stevens et al., depicted an increase in stability near to 50% when amino acids and sucrose were used in combination. Out of all tests carried on amino acids it was concluded that serine in combination with sucrose showed enhanced stability where glutamic acid was shown to portray negative outcome (Bjelošević et al. 2020).

Patel et al., conducted a study of mAb formulations and used combinations of various amino acids with or without addition of sucrose. It was found that arginine, lysine and proline showed pragmatic effect on protein stability even without sucrose addition. Whereas, alanine and glycine required sucrose addition to stabilise the protein. It was also perceived from studies that combination of arginine, alanine and glycine showed reduction in reconstitution time but sucrose when was used alone showed exactly opposite effects (Bjelošević et al. 2020).

Amalgamation of mannitol and amino acids (glycine/lysine) with sucrose resulted in depression in T_g which increased with addition of substances. This phenomenon was attributed to the nature of amino acids exhibiting low T_g but the depression in case of mannitol was not explainable (Lueckel et al. 1998).

Computational Models and Simulation: Role in Selection of Cryoprotectants

Cryoprotectants like chitosan and its derivatives along with varied saccharides have been studied for their ability to maintain the functionality of proteins and peptides. These stabilize proteins and prevent water withdrawal from protein. The molecules functionalize in a specific pattern by increasing the surface tension of water and preventing protein solubility. Hypothesis has been formulated for understanding the underlying mechanism of their action and studies have revealed how the molecular level interactions can help in further exploration. Different classes of cryoprotectants differ in their molecular properties, for instance, the active functional groups present in them, the type of interaction and spatial arrangements. These molecular properties in turn have shown to affect the physicochemical properties of cryoprotectants which contribute to its mechanistic action and performance. Proteins and peptides are susceptible to temperature changes which affect their crystal structure, stability and integration. These molecules are integrated chains of many small polar molecules (amino acids). Subsequently, the functional groups, molecular arrangement, partial charges and sequence of amino acids affect the properties of proteins and peptide domains present in their quaternary structures. Therefore, an insight into the molecular properties of proteins is of foremost importance in selection of cryoprotectants.

Understanding Cryoprotectant-Water Mixture Using Molecular Dynamics and Neutron Diffraction

A series of molecular dynamic simulations has revealed that, in a mixture of water and cryoprotectants, particularly polyhydroxy cryoprotectants (e.g., alcohols and sugars), the hydrogen-bonding interactions between cryoprotectant and water contribute to the diminution of bulk water and the establishment of the hydration shell of the cryoprotectant as the solute concentration increases. The transition from solvent dominant system to a solute dominant system was studied by increasing the solute concentration in the simulation model. The hydrogen bond formed by a water molecule remains constant, however, as the polyhydroxy cryoprotectant is changed, the number of hydroxyl groups also changes. Consequently, the change defines the ability of the cryoprotectant to form hydrogen bonds in number. The formation of an extended solute network may have various implications for the stabilization and protection of biological materials. On one hand, the large sugar clusters have been suggested to increase the mixture's resistance to shear deformation and mechanical stability (Molinero et al. 2003). Initially, the simulations were carried at room temperature, later when combined with neutron diffraction studies demonstrated that the characteristic nano segregation between a polyalcoholic cryoprotectant and water persist at low temperature. Similarly, the investigations of saccharide cryoprotectant and water mixture at low temperature revealed that the bound and free portions of water and clustering of saccharide molecule existed and depressed the molecular mobility at 150 K (Weng et al. 2016). Depending on the extent of heterogeneity of the cryoprotectant–water mixture and self-diffusion coefficient, the slower cryoprotectant molecules may act as roadblocks to prevent the local gathering of water molecules to form ice embryos, which can delay ice nucleation and cause the mixture to favour glass formation (Weng et al. 2019).

Simulation Models for Cryoprotectant-Proteins/Peptides Mixture Using Molecular Dynamics and Homology Modelling

Molecular dynamics and homology modelling were able to predict the suitability of cryoprotectant. Myosin as a template protein and saccharides as cryoprotectants were used for the simulation studies, where the three-dimensional structure of myosin was built using Modeller software. Molecular dynamic simulations between myosin and saccharides were performed using Amber 12 software. Energy optimizations were carried using Amber ff03 and Amber gaff force fields and subsequently equilibrated at 300 K. During simulation, all bonds involving hydrogen atoms were constrained within the linear constraint solver (LINCS) algorithm. Duncan's test was used to determine significance in difference. The root-mean-squared deviation (RMSD) parameter measures the overall changes in conformation from the initial or any other reference structure. RMSD values with respect to myosin in the water system illustrated that the structures in the trajectories significantly differed from that in the saccharide system. However, the calculated RMSD values of the myosin structures when the saccharides were incorporated showed significantly lower fluctuations than those observed in the simulations that excluded the saccharides (Edelman et al. 2015). These observations indicate that the inclusion of the saccharides, which presumably affects the structure of myosin, the distribution of water molecular around protein molecules and/or the hydrogen bonds between the hydroxyl groups of saccharides and proteins, led to a decrease in RMSD fluctuations and a better protection of protein stability (Zhang et al. 2018).

Combining Simulation Results from Cryoprotectant-Water Mixture and Cryoprotectant-Protein Mixtures

Molecular dynamic study of cryoprotectant-water mixture provides information of the structural changes and spatial arrangement of both water molecule and cryoprotectants. It is predominantly observed how the surface tension of water can be affected by the hydrogen bond formation between molecules of water and cryoprotectant. In addition, the cluster formation of cryoprotectants also restricts water molecules to come together, thereby affecting nucleation. Avoidance of water crystal formation is an important factor when considering freeze drying. This protective action of cryoprotectants can be owed to the aforementioned mechanism. Moreover, when proteins and peptides are considered, the interaction with cryoprotectant and conformational changes are regarded as grounds for selection of cryoprotectants. Therefore, a prediction model combining both the molecular dynamic simulations can provide a more affirmative predictability in selection of cryoprotectants for proteins and peptides (Fig. 7).

Conclusions

The field of synthetic peptide vaccines is ever increasing and is very crucial in the area of immunology. Synthetic peptide vaccines have gained importance because of reduced

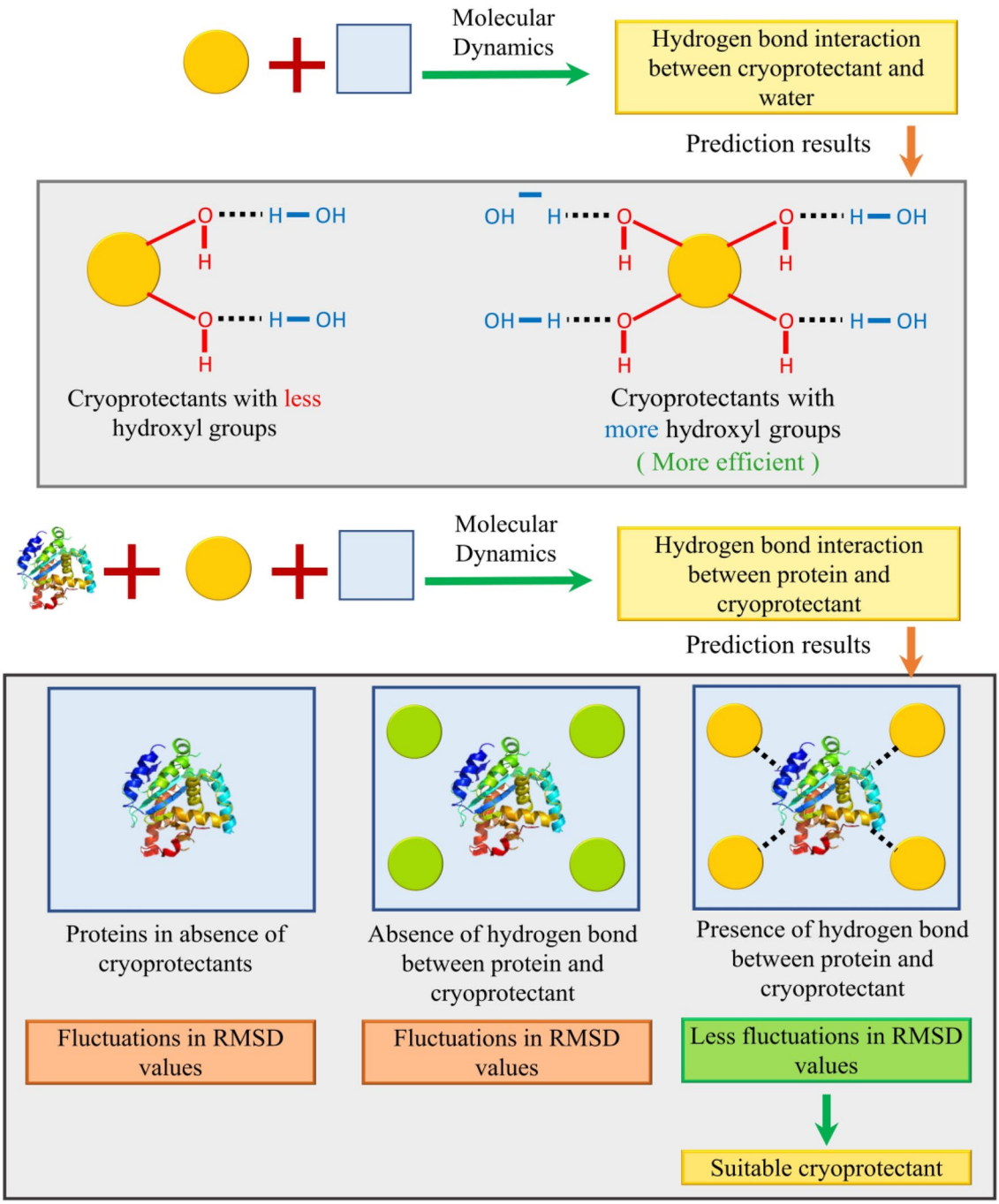

Fig. 7 Simulation and prediction representation of hydrogen bonding (black) capacity of cryoprotectants (yellow) in water (blue) and between cryoprotectants (green and yellow) and protein using molecular dynamics. Surface tension of water is affected by the hydrogen bond formation between molecules of water and cryoprotectant. Moreover, the cluster formation of cryoprotectants also restricts water molecules to come together, thereby affecting nucleation. Avoidance of water crystal formation is an important factor during freeze drying (Color figure online)

time and increased ease of production coupled with better immune protection that they provide. The major issue of stability faced by peptide vaccines can be solved by employing processes like lyophilization and exploiting the capabilities of cryoprotectants. Despite this, the field of synthetic vaccines is not utilized to its full potential. Technology like molecular dynamics can be used to study the interactions between cryoprotectants and peptides at the molecular level. This study can give useful insights into the actual manner in which cryoprotectants stabilize peptides and help in

maintaining their immunogenicity. Such studies will help the formulation scientists to arrive to a stable formula for the peptide vaccines, including all the required excipients, and will fasten the process of their development. The power of technological advancements like molecular docking, artificial intelligence (AI), 3D printing, etc. can be harnessed to aid the synthesis and in providing stability to the synthetic peptide vaccines. This will open up new field of opportunities for all the researchers.

Declarations

Conflict of interest The authors declare that they have no known competing financial interest.

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