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Challenges in peptide solubilization – amyloids case study

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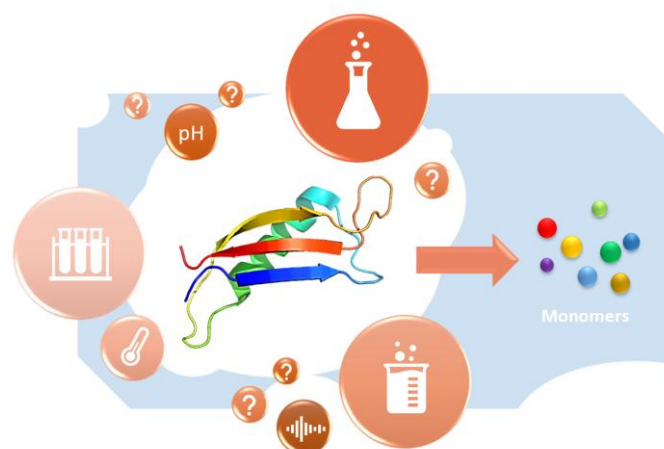
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ABSTRACT

In this Review, we provide insights into the peptide solubilization problem through the aggregation phenomenon and critically examine various available solubilization protocols. We focus on amyloids, which are particularly difficult to dissolve and handle.

GRAPHICAL ABSTRACT



KEYWORDS

aggregation, “difficult sequences”, monomerization, solubility

INTRODUCTION

Peptide science has been a rapidly growing research field due to the enormous potential application of these biocompatible and bioactive molecules [1]. A wide diversity of natural and modified peptides has been obtained and studied, covering multiple therapeutic areas [2]. Many factors limit the widespread use of peptides in medicine, such as short half-life and low cell membrane permeability [3] and low solubility is among the most common problems that hamper drug development in the early stages of research [2]. Solubility is a crucial, albeit poorly understood, feature that determines peptide behavior. For example, low solubility results in the formation of peptide aggregates that affect the biological activity of peptides, which is associated with many diseases [4][5]. Several approaches have been reported to overcome problems caused by the low solubility of therapeutic peptides, that is, mutating the sequence by adding charged residues [6], attaching polymers [7], or performing encapsulation [8]; however, these strategies are complex and do not always generate the desired results. One of the areas most affected by low solubility is the study of amyloids, which are highly insoluble aggregates of proteins and peptides that can form plaques in areas such as nerve tissues. For this reason, amyloids are associated with a number of serious diseases with unclear causes, including Alzheimer's disease, Parkinson's disease, and type 2 diabetes [9][10][11]. Amyloids are formed during the abnormal aggregation process when individual species self-associate and form higher-order structures that are stabilized by a wide range of intermolecular interactions, such as hydrophobic effects, van der Waals interactions, π - π stacking, and hydrogen bonds [4][12][13].

Solubility can be defined as the ability of a chemical compound to dissolve in a solvent and form a homogeneous solution [14]. Solubilization also involves the breaking of noncovalent interactions between molecules in aggregates. In amyloid studies, the term "monomerization" is often used to describe the dissolution of peptides to a pure monomeric state [15], as shown in Figure 1. This term refers to a single peptide or protein molecule/unit, regardless of structural state or solubility, that undergoes a further aggregation process due to the cooperative series of self-interaction events [16][17]. Monomerization is a problematic but crucial step in several studies, such as kinetic studies and structural and functional examinations. Monomerization can be achieved through various approaches, including the use of chemical agents (such as denaturants or surfactants) that disrupt the intermolecular interactions that stabilize the aggregate structure. Other methods involve altering environmental conditions, such as pH, ionic strength, or temperature, to promote the dissociation of aggregates into monomers.

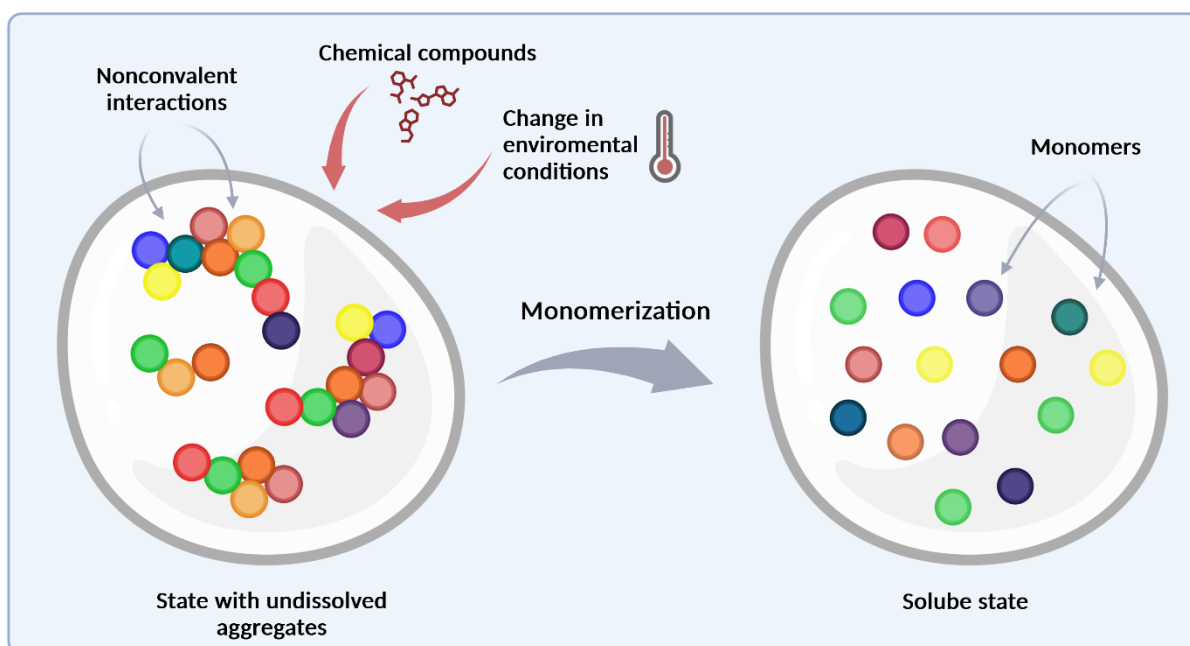


Figure 1 Peptide monomerization scheme.

In addition, amyloids cause problems in the synthesis and purification stages because they quickly aggregate and are difficult to solubilize in aqueous solutions [18][19]. New articles are continually being published that contain novel protocols for the synthesis, purification, and solubilization of amyloids [20][21][22][23], demonstrating that the previously proposed methods are not universal. In this minireview, we present the results of literature research on the challenges encountered in peptide solubilization in aqueous and organic solutions. Although peptides are built up with amino acids like proteins, the properties and working procedures are fundamentally different for those molecules. Therefore, in this paper, we focused only on peptides, especially amyloids, which are extremely demanding in terms of dissolution. We propose a critical view of the proposed methods on the basis of our own experience. Moreover, we endeavor to propose several approaches and tips to overcome solubility problems.

OBTAINING PEPTIDES AND STUDYING THEIR SOLUBILITY

Researchers have obtained and modified peptides by chemical and biological techniques. Biological methods include enzymatic hydrolysis, microbial fermentation, and a recombinant approach [24][25]. Chemical synthesis, especially solid phase peptide synthesis (SPPS) [26], has recently become very popular due to the application of microwave-supported automated synthesizers, which facilitate and accelerate the peptide production process and allow the acquisition of sequences with noncanonical amino acids, which is not possible when working with bacteria. Biological methods are advantageous because very long sequences that are not achievable for automated SPPS can be obtained. Interestingly, the selected method can affect the physicochemical properties of the obtained peptides;

for example, recombinant amyloid beta (A β) aggregates three times faster than the synthetic version and is more toxic [27][28]. Additionally, some peptides tend to aggregate in the resin, which is a problem in the SPPS technique [29]. Aggregation leads to incomplete solvation of the peptide-resin complex, reduced reagent penetration, and matrix shrinkage. As a result, synthesis fails in acylation and/or deprotection reactions. Regardless of the chosen method, there are some peptides that are more difficult to obtain than others, such as amyloids, which are called 'difficult sequences'. This is because sequences that are especially susceptible to aggregation are enriched with hydrophobic residues (alanine (Ala), valine (Val), isoleucine (Ile)), sequences containing amino acids that form intrachain hydrogen bonds (glutamine (Gln), serine (Ser), threonine (Thr)) [18][30] and long sequences containing more than 20 amino acids [18].

Moreover, C- and N-terminal modifications can have a significant impact on peptide assembly dynamics and aggregate morphology [31]. For example, it was shown that hIAPP(20–29) without terminal modifications formed flat fibrils, C-terminal amidation induced the formation of twisted fibrils, and N-terminal acetylation completely inhibited fibrillation [32]. During production, peptides can also be contaminated with byproducts of synthesis or cleavage. The most common contaminants are salts of trifluoroacetic acid (TFA) or hydrogen fluoride (HF), along with chemical scavengers used during synthesis or enzymes and other peptides used in recombination techniques. Contaminants can complicate preparation of peptide stock solutions in physiological buffers and lead to solubility issues [27][24][4]. In addition, contaminants can change the biophysical and biological behavior of the peptide; for example, the growth of amyloid fibrils can be slowed [27][24][28]. Additionally, if aggregates form in the resin during synthesis, purification of peptides is particularly problematic because aggregates are resistant to many denaturing agents, so they are difficult to dissolve [18][33]. For instance, high-performance liquid chromatography (HPLC) systems can be plugged by the peptides precipitating on the (pre)column.

Properly storing peptides is also important because repeated freezing and thawing can cause chemical degradation, conformational changes, or even promote or disrupt aggregation processes. These changes thus jeopardize the repeatability of experimental results [27][34][35]. Additionally, lyophilized powders should be protected from light, especially if the peptides contain aromatic rings and should be sealed [36]. The material should be stored at -20 °C for shorter times [34] and at -80 °C [27][10] for prolonged storage. Frequent freezing and thawing during the performance of experimental procedures is not recommended because the process influences peptide stability [37][38].

The solubility of peptides is crucial to their biological activity. Peptides often interact with other molecules, such as enzymes, receptors, or other proteins, to perform their biological functions [37]. Properly dissolved peptides are freely available and well dispersed in a solvent, allowing efficient molecular recognition and binding [5][11][39]. Furthermore, solubility is important for bioavailability and pharmacokinetics. High solubility simplifies the formulation process, allowing better control over dosage and delivery methods, such as oral, injectable, or transdermal routes [37]. Moreover, well-

soluble peptide-based drugs are more evenly distributed and absorbed more efficiently in biological fluids. Drugs with good solubility can move more easily in biological systems through cell membranes or body fluids, allowing them to reach their targets. Therefore, proper solubility has a strong influence on the effectiveness, delivery, absorption, and distribution of peptide-based drugs in the body, which are crucial to achieving the desired therapeutic effects [14][13].

Proper solubilization of peptides is also crucial in experimental techniques because peptides that readily dissolve are easier to handle, manipulate, and analyze compared to those that are insoluble. A technique that requires monomeric peptides is a fluorometric method using the dye thioflavin T (ThT). Through this technique, the progress of aggregation can be tracked. Only accurate monomerization allows approximation of a specific function (most often sigmoidal) to the obtained results, allowing for correct analysis of the data, determination of kinetic parameters, and selection of the appropriate aggregation mechanism [15][27]. The presence in solution of aggregates of different sizes that have not been dissolved initiates aggregation because the aggregates present act as seeds. As a result, aggregation kinetics are falsely accelerated [40][27][34][41][19][10]. Other examples of techniques that require good peptide solubility include nuclear magnetic resonance spectroscopy. Recording NMR spectra under suitable conditions requires stability of the peptide over time at the chosen pH and temperature, which also indicates that they should not aggregate [42]. Moreover, in X-ray crystallography, peptide solubility is crucial to obtain well-grown crystals that can be further analyzed [43]. However, achieving an ideal monomeric sample necessitates careful handling under specific nonaggregation conditions. These conditions often involve the presence of organic solvents, high pH levels, or the use of chaotropic agents [19], which will be discussed in the following section.

It is crucial to consider the abovementioned factors, as they can significantly affect the reproducibility of experiments and influence the quantity, quality, and physicochemical properties of the resulting peptides [27][34][44][19][4]. The process of solubilizing and converting peptides to their monomeric form can be challenging, especially when dealing with amyloids and other peptides with low solubility [9][41], and these processes are essential to ensure proper functioning of peptide-based therapeutics and obtain reliable data in the experiments. However, it is imperative to overcome these challenges to ensure that the peptide-based therapeutics properly function and to obtain reliable data in experimental studies.

INTRINSIC AND EXTRINSIC FACTORS THAT AFFECT PEPTIDE AGGREGATION

The solubility of peptides is determined by their intrinsic factors; therefore, the propensity of the peptide sequence to aggregate, directly related to the hydrophobicity of the peptide and its secondary structure preferences (β -sheets are more prone to aggregation than helices) [4]. Thus, aggregation is a complex process influenced by many internal and external factors that must be considered when studying this phenomenon. These factors are shown in Figure 2.

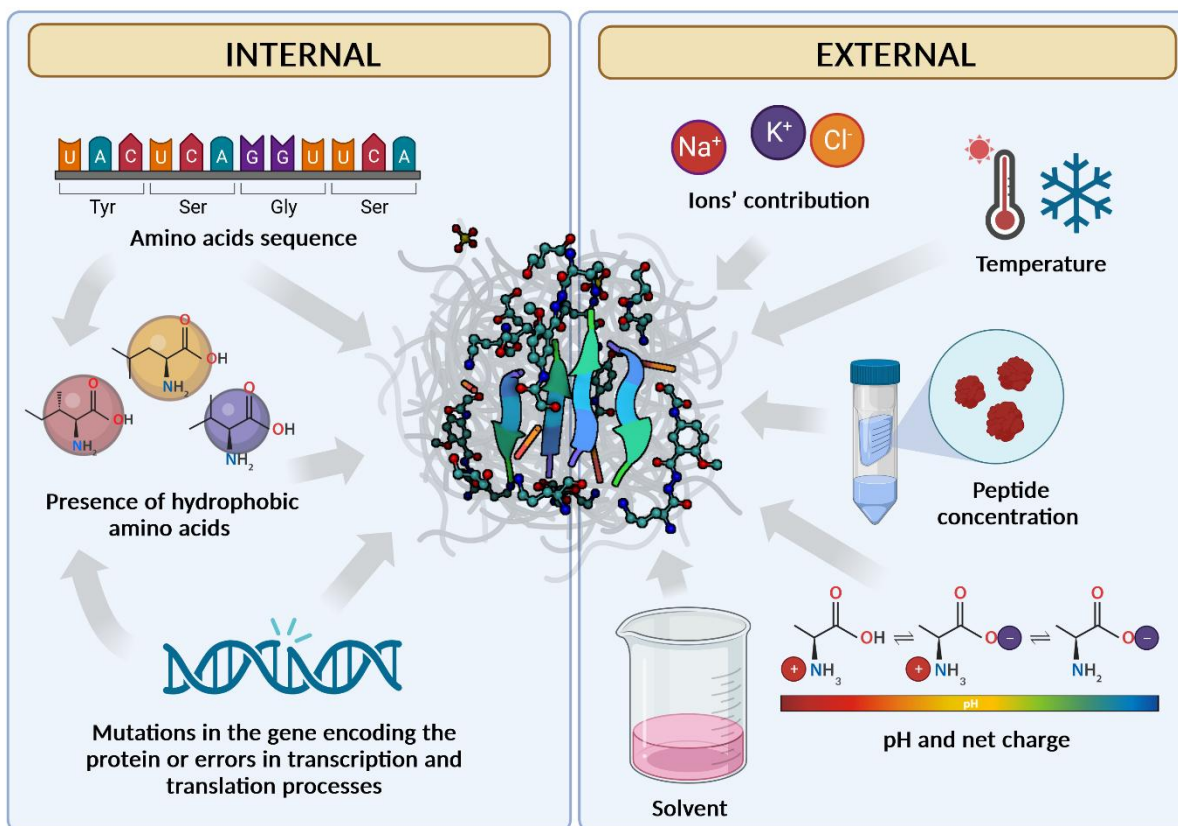


Figure 2 Intrinsic and extrinsic factors affecting peptide aggregation.

The sequence, both in terms of the amino acid composition and the order of the residues, is the main intrinsic factor that influences the aggregation propensity of peptides. In general, the presence of hydrophobic amino acids in the sequence increases the tendency to aggregate, as it reduces the total net (overall) charge of the peptide [45]. Several bioinformatic methods have been developed for the identification of amyloidogenic fragments of peptides and proteins to predict the so-called aggregation-prone regions (APRs) [46], which are likely to trigger the aggregation process. The algorithms consider not only amino acid sequence but also total hydrophobicity, charge state, secondary structure propensity, etc., to estimate the aggregation propensity of the peptide [47][48]. Studies on the effects of mutations on aggregation propensity [49][50][51][52][53] have shown that even a single mutation can favor aggregation [49][50]. On the other hand, gatekeeper residues have been identified [54][55] that prevent the aggregation process from occurring. For example, the gatekeepers in the CsgA protein are aspartic acid (Asp) and glycine (Gly) residues [56].

The crucial extrinsic factor that influences the aggregation process is concentration. In general, higher concentrations of peptides increase the likelihood of the formation of aggregation nuclei, which can accelerate the entire process [57]. The most important characteristic of the nucleation-dependent

polymerization mechanism (the most frequent model, in which the aggregation process is controlled by peptide/protein concentration and time) is that no peptide aggregation occurs below a critical concentration (c_R) [58]. However, the c_R is different for each peptide and depends on the general conditions of the solution [59]. This can be interpreted as the minimal concentration of peptide needed to form nuclei, meaning that below c_R , peptide species are in monomeric forms. For example, the R2 and R4 fragments of the CsgA protein do not fibrillate at a concentration of 0.2 mg/ml. However, fibrils were observed at a concentration of 2 mg/ml [60]. Time is an important factor that influences aggregation. Most peptides have a natural tendency to aggregate (hence the great interest in self-associating peptide-based nanomaterials); therefore, it is postulated that the aggregation process is just a matter of time (second vs. even years) [61][62].

In terms of the general solution conditions, the pH of the solution has the most significant impact on the aggregation process. This is directly related to the fact that peptides are amphoteric compounds because of the presence of amino and carboxyl groups in the side chains, so they behave like acids or bases, depending on the pH of the solution. Thus, when the solubility of a peptide is evaluated, the amount and type of charged amino acids, i.e., lysine (Lys), arginine (Arg), and glutamic acid (Glu) in the sequence are considered. Generally, the higher the net charge of the peptides is, the slower the aggregation, and the lower the net charge is, the greater the propensity for aggregation [4]. Moreover, each peptide has a characteristic pH value, called an isoelectric point (pI), in which it carries no net electrical charge or is electrically neutral in the statistical mean, so the number of positive and negative charges on the surface of the molecule balances each other. At a pH below its pI, peptides carry a net positive charge, and at a pH above its pI, peptides are negatively charged. At the pI, the solubility of peptides in water is the lowest, and their ability to aggregate is the highest [5][24]. In general, peptides with more charged residues should be more soluble in water. Therefore, the high content of hydrophobic residues reduces the solubility of the peptide in aqueous solution [30][14]. Moreover, fibril formation has been shown to be modulated by pH; for example, human islet amyloid polypeptide (hIAPP) and insulin are in monomeric forms only at acidic pH [63][64]. Amyloids usually possess a low net charge [65], but they are still closely related to the pH of the solution.

The addition of any acid, base, or salt introduces additional ions to the solution, shifting the acid–base balance. As a consequence, the pH of the solution can be changed, causing a modulation in the charge of the peptide [57]. The addition of salts can stabilize or destabilize the peptides and influence the aggregation process, since both cations and anions present in the solution can electrostatically interact with the peptide surface, which can affect not only conformation but also the kinetics and morphology of the formed fibrils. It was shown that the nature and concentration of ions can control the formation of a large number of amyloid-like forms from the nanoscale to the microscale [66]. The Hofmeister series categorizes ions according to their kosmotropic and chaotropic properties [67]. Contrary to kosmotrope ions (water structure makers), which possess a propensity to stabilize the native fold, leading to the salt-out effect, chaotrope ions (water structure breakers) facilitate denaturation and

unfolding [68]. Salts such as NaNO_3 , Na_2SO_4 , Na_2HPO_4 , and NaClO_4 were shown to influence the rate constant of the aggregation process of human insulin and chicken egg white lysozymes [66]. On the other hand, NaCl is a safe choice for studies due to the lack of strong specific Na^+ and Cl^- interactions with most peptides [57]. The charge and concentration of ions present in the solution translate into the ionic strength, which affects the stability of peptides and aggregates, their formation rate, and morphology. [66]. Ionic strength has been shown to have an impact on the rate of aggregation [4], the lag phase and the growth of amyloid fibrils [69].

A strategy based on the addition of acid or base to the working solution to facilitate the dissolution of the peptide is often used [63][64][34][36]. However, it is often necessary to create a natural physiological environment for further studies, which requires a transition from acidic or alkaline to neutral pH. During this adjustment, the pH of the solution passes through the pI, at which the aggregation propensity is maximal, and solubility is minimal, which can result in the conversion of monomers into a polydisperse population of low-order oligomers and higher-order polymers. This population has irreproducible assembly behavior characterized by significant pH-dependent morphological and kinetic differences in fibril formation [24]. For some peptides (e.g., A β), this can be avoided by alkaline solvation, resulting in higher monomeric peptide yields and a low aggregate content [27]. To mimic physiological conditions and control pH, various buffers with different buffering capacities have been proposed for peptide studies [4][70][71].

Another important factor is temperature, which is one of the key environmental factors that alters the behavior of particles in solution, influencing vibrational motions and the overall diffusion in solution [72]. Consequently, a change in the temperature of a solution studied can destabilize the peptides and trigger an aggregation process. In general, increasing the temperature accelerates the aggregation process due to the creation of additional hydrophobic interactions [73] and increased diffusion of peptide molecules [74] and influences the aggregation kinetics [75]. For example, even a small increase in temperature, from 37 °C to 39 °C, can promote the aggregation of A β 40 and A β 42 [76].

Interaction with other peptides and proteins is one of the factors that should also be considered, as Parkinson's, Alzheimer's, or type 2 diabetes have been linked to the aggregation of different amyloid proteins [77]. Several *in vitro* and *in vivo* studies have shown that one amyloid can trigger the aggregation of another in a phenomenon called cross-seeding (or cross-talk) [78][79]. The interaction between, for instance, A β and hIAPP [80], α -synuclein [81], and tau [82]. It seems that cross-seeding is possible mainly between proteins and peptides with a high percentage of identical residues (identity) and a high percentage of residues conserved with similar physicochemical properties (similarity). Interestingly, cross-talk can also lead to the inhibition of aggregation [83][84]. Recently, the effect called the brain-gut axis has been described [85], where amyloids in the microbiome could affect the progression of neurodegenerative diseases by influencing the rate of aggregation of pathological amyloidogenic proteins [86]. The influence of the parameters on the aggregation process described above should be treated with caution, as there are exceptions to all of them [4]. Additionally, many

other factors, such as pressure [87], irradiation [88], stirring speed [89], and interaction with the test tube [90][4][72], can influence aggregation and, consequently, solubility of the peptide. It is necessary to bear in mind that all the abovementioned factors can affect the reproducibility of the experiments, so a detailed description of experimental conditions is essential and an integral part of the conducted studies.

GENERAL RULES AND PROTOCOLS GOVERNING SOLUBILITY OF PEPTIDES

As mentioned above, the solubilization of peptides to their monomeric form is crucial in the study of peptide aggregation, synthesis, purification and more [15][18][27][34][19]. There is no universal technique for performing this operation, even for well-studied peptides such as A β [19][24]. Additionally, many peptide manufacturers on their websites recommend certain solvents for peptides with specific physicochemical characteristics, that is, net charge of the peptide, pI, percentage of hydrophobic residues, and number of residues [91][36].

Crucial factors that must be taken into account when dealing with the solubility problem are the pH of the solution and the pI of the peptide. There exist databases with experimentally measured pI of peptides [92][93], containing information about predicted pI [94], and pI calculators [95]. There are also a few other parameters, such as net charge, average hydrophilicity, and grand hydrophathy indices GRAVY (Table S1), that help assess the physicochemical nature of peptides and thus are related to their solubility. The values of these parameters can be easily determined using online peptide physicochemical property calculators, such as [96][97][98][99][100][101][102]. However, the prediction of these parameters does not necessarily bring about solutions to solubility problems. There are also quite a few predictors of protein solubility, such as CamSol [103], DeepSol [104], DeepSoluE [5], PaRSnIP [105], Protein-Sol [106], PROSO II [107], SODA [108], SOLart [109], and SWI [110]. These methods use a variety of approaches, including machine learning [5][13]. Despite numerous predictors of solubility, their effectiveness is unsatisfactory in the context of the current significant increase in the number of protein sequences available [5]. In addition to information on dissolution, aggregation, and physicochemical properties, some calculators also include information on ease of synthesis and purification [101]. It is worth remembering that individual calculators often use different algorithms and other values from the literature, so the resulting parameters may differ.

In general, peptides with short sequences (< 5 amino acids) and containing >25% charged residues (Asp, Glu, Arg, Lys, histidine (His)) and < 25% hydrophobic residues (Val, Ile, tyrosine (Tyr), leucine (Leu), phenylalanine (Phe), methionine (Met), tryptophan (Trp)) are usually soluble in water or aqueous solutions [36][111][91][112]. Typically, the solvent is distilled water or filtered high purity water to eliminate all nanoparticles and impurities [24][111]. However, short peptides can be problematic if the entire sequence consists of hydrophobic amino acids. In such a case, organic solvents must be applied [91], which are described in detail later in this article.

If the peptide is not soluble in aqueous solution and is nonhydrophobic (<50% hydrophobic residues), acidic (net charge < 0) and/or the total number of charges of the peptide at pH = 7 is greater than 25% of the total number of residues [113]), various bases are used, such as sodium hydroxide (NaOH), ammonium hydroxide (NH₃·H₂O) [24][41][27][34] or ammonium bicarbonate (NH₄HCO₃). NaOH is usually used at a concentration of 2-50 mM (pH 11-12.5) [112][24], NH₃·H₂O as 0.02-25% [36][91][114][115], and NH₄HCO₃ as 0.1 M [113][112]. Studies have shown that NaOH and NH₃·H₂O were the most effective in both preventing aggregation and disaggregating fibrillar structures. Furthermore, NH₃·H₂O is compatible with most analytical methods. The use of barium hydroxide (Ba(OH)₂) and calcium hydroxide (Ca(OH)₂) has also been reported [41]. Strong bases should not be used if the peptides contain cysteine (Cys) because disulfide bonds can form at an alkaline pH [36][116]. Therefore, such peptides are best dissolved in degassed solvents, for example, buffers pH < 7, diluted acetic acid (CH₃COOH) or 0.1% TFA in aqueous acetonitrile (ACN) [112]. It is worth remembering that the stability of the peptide solution deteriorates when the pH is 8 or higher. Therefore, it is recommended to keep the solutions in the pH range of 4-6 [117].

If the peptide is not soluble in aqueous solution, is nonhydrophobic and is basic (net charge > 0 [113]), various acids are used, mainly hydrochloric acid (HCl), formic acid (HCOOH) and CH₃COOH [27][34][24]. Some manufacturers recommend using a small amount of 10-30% CH₃COOH as a first step [111][113][112]. If this does not help, the addition of 0.1% TFA or 0.1% HCOOH in the buffer is suggested [91]. However, the addition of HCOOH can result in the formylation of Ser [34].

Finally, for neutral peptides (net charge = 0), whose total number of charges is greater than 25% of the total number of residues, the strategy described for acidic peptides is recommended [113]. Importantly, a gel can form when a base is added. Generally, this gel will only respond to dilution with higher amounts of distilled, deionized water, along with gentle vortexing [36].

If the peptide has fewer charged residues, it may be necessary to use organic solvents. These can make peptides that are not soluble in aqueous buffers at least temporarily soluble. As a first step, it is best to add ACN, which, when added to the water solution, disrupts aggregate formation by solubilizing hydrophobic regions as a result of reduced solvent polarity. Moreover, the addition of ACN to water lowers the dielectric constant, so the electrostatic repulsive interactions present at low pH are enhanced [118]. Methanol, isopropyl alcohol and ethanol are also recommended [10].

If the above methods fail, dimethylsulfoxide (DMSO) or dimethylformamide (DMF) is often suggested [119][111][112], but not when the peptide contains Cys, Trp and Met because they are susceptible to oxidation [36][91]. Instead, it is recommended to dissolve Cys-, Met-, and Trp-containing peptides in oxygen-free buffers [91]. It is worth noting that DMSO (and DMF) is difficult to remove by lyophilization processes [35].

Organic compounds are also used for highly hydrophobic peptides (> 50% hydrophobic residues [36][91]) and/or if the total number of charges of the peptide is less than 10% of the total number of

residues [113]. Peptides that are prone to aggregation may require strong denaturants, e.g., >6 M urea, 6 M guanidine-HCl [36] or a mixture of 6 M urea with 20% CH₃COOH [91]. These compounds facilitate the breakdown of hydrophobic interactions or reduce the "gelation" of peptides by disrupting the hydrogen bonding network [113]. Furthermore, they can interfere with most biological systems (e.g., reduce the aggregation rate), so their use is quite limited [91].

In addition to the organic solvents mentioned above (ethanol, methanol, isopropanol, ACN, DMSO, DMF), hexafluoroisopropanol (HFIP), TFA, and tetrafluoroethene (TFE) are also applied to dissolve peptides [24][27][10]. They are usually used at a 100% concentration and then diluted with water or buffers to the desired concentration [91]. From a peptide science perspective, the order of preference is as follows: HFIP > TFE > DMSO > TFA. However, application, working buffer, and experimental setup must also be taken into account [27]. For peptides >75% hydrophobic, highly concentrated TFA or HCOOH is recommended [36]. The chemical properties of the selected organic solvents recommended for the solubilization of the peptides are shown in Table S2.

It is worth remembering that when using HFIP, most protocols describe preincubation of the peptide in a concentrated (100%) solvent, which is then evaporated. In some cases, HFIP is not removed prior to dissolution in aqueous buffer. However, this protocol is believed to be flawed, as there is extensive evidence that low concentrations of HFIP are ineffective in dissociating oligomers and can enhance fiber formation [41]. Similar procedures are recommended for TFA. Methods in which both solvents are mixed in a 1:1 ratio to improve solubility have also been described [10]. Furthermore, a mixture of TFE or HFIP and trichloromethane (TCM) or dichloromethane (DCM) has been shown to be more efficient in solubilizing peptides than each solvent alone [112].

Once the peptides have been dissolved and reduced to a monomeric form, research can begin. When the aggregation process of amyloid peptides is studied, the next step is to create conditions suitable for aggregation. The buffers that are used most are Tris, HEPES, or PBS at different pH values [24][36][91][120][41][121]. In turn, in pharmacology, solvents such as acetate, citrate, phosphate, and Tris [4] are often used. It is worth mentioning that incubation of peptides in buffers under physiological conditions creates excellent conditions for aggregate formation and bacterial growth. In some peptide aggregation assays, dyes such as ThT or Congo Red (CR) can bind to them, giving false-positive results. To inhibit their growth, 0.05% [34] (or 0.01% [24]) sodium azide is added to solutions, the incubation temperature is reduced to 4 °C, or H₂O is replaced by D₂O in buffers [34].

The above information is summarized as an algorithm for the solubilization of peptides based on their physicochemical nature (Figure 3).

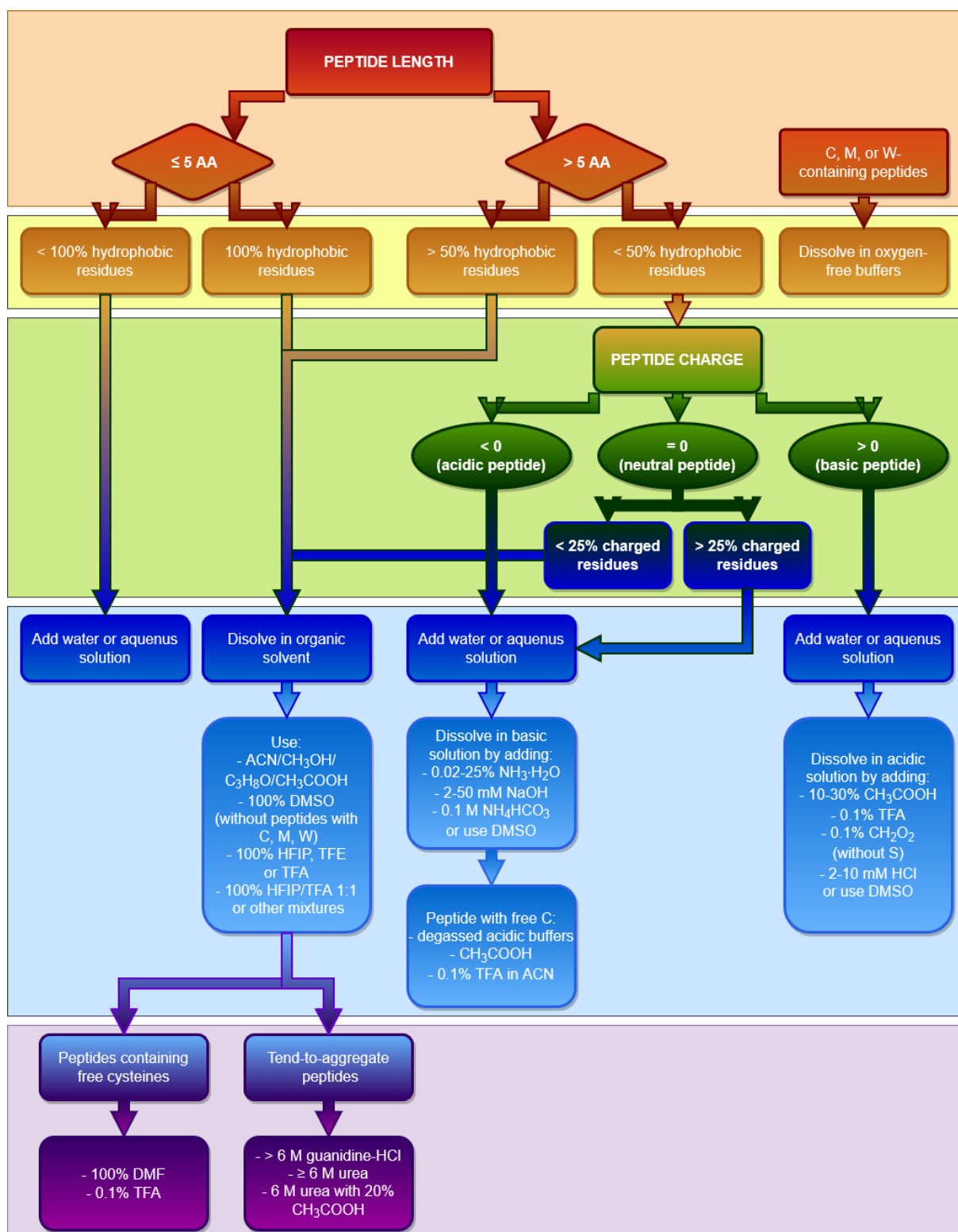


Figure 3 Scheme for handling peptides with specific physicochemical properties for solubilization.

Treating peptides with strong bases and other solvents is effective because it reduces the number and size of existing aggregates. However, this approach alone is not always sufficient to ensure the monodispersity and homogeneity of the samples [27][41], as aggregates can form when peptides dissolve. Most oligomerization reactions are in dynamic equilibrium, so oligomeric species can form

again after their removal [27]. Thus, a second preparation step is often necessary to obtain a fully disaggregated solution. For example, filtration and/or ultracentrifugation are applied to eliminate higher mass aggregates [27][24]. Using filters with a porosity of 20 nm or with a 10 kDa exclusion limit is sufficient and yields solutions free of preaggregates, leaving monomers and dimers in solution. Filters with a diameter of 0.22 μm are also often used in experiments. These remove particles larger than 200 nm, which is a typical size of fibrillar aggregate. A disadvantage of the above methods is the loss of material, as it can, for example, remain in the tube or stick to the filter membrane. Therefore, it is recommended to rinse the filters with ultrapure water prior to use and then add buffer [27]. In addition, some fibrils can pass through pores 20 nm in diameter. Furthermore, most commercially available syringe filters are prepared with wetting agents that absorb well below 220 nm, which may interfere with measurements in some studies. Therefore, filters should be rinsed with DDI water prior to sample filtration [34]. To evaluate the effectiveness of the methods used and the degree of dissolution of the peptide, it is useful to perform a visual assessment. If the solution has gelled, is cloudy, or contains visible particles, this indicates that the peptide has not been completely dissolved but is suspended. The clear solution suggests that the procedure used is correct and that the peptide is ready for further testing [112][113]. If this is not the case, sonication is often recommended. It increases solubilization by breaking the test peptide into smaller molecules using ultrasound [122][123]. However, it has been shown to affect the kinetics of aggregation and the morphology of the resulting fibrils [124][125]. If precipitation occurs, gentle warming of the peptide solution would be helpful, but excessive warming of the sample must be avoided [91]. In addition, more sophisticated separation methods, such as size-exclusion chromatography (SEC) [27][24] or asymmetric flow field-flow fractionation with multiangle light scattering (AF4-MALS) [23], are used to separate the fraction with desired masses. The use of more advanced methods, compared with filtration, allows the collection of different populations of oligomers, including protofibrils, as well as relatively pure populations of monomers, dimers, and trimers. However, they are more time-consuming techniques and require appropriate equipment [19][34][24][27]. Oligomer size distributions can be verified using dynamic light scattering (DLS) [126] or mass spectrometry with ion mobility (IM-MS) [127]. In addition, atomic force microscopy (AFM) and transmission electron microscopy (TEM) can be applied to examine the morphology and size of the sample [128][129][130].

DISCUSSION ON PEPTIDE SOLUBILIZATION PROTOCOLS FOR A β , INSULIN AND PSM α S

Apart from the general methods described above recommended for the solubilization of peptides, dozens of elaborate protocols focused on specific sequences, often with important biological functions, such as amyloid beta, insulin, and phenol-soluble modulin peptides, have been reported. In Table S3, protocols found in the literature were compiled that describe the dissolution and monomerization of

the chosen peptides, including methods for obtaining specific oligomers and small aggregates. A short description of each peptide is given in Table S3.

In general, the collected protocols represent diverse approaches for the dissolution of each peptide, including various compositions of buffers, organic solvents, and additional steps. To assess the validity and effectiveness of a particular approach, we must first focus on the sequence amino acid composition (summarized in Table S4) and the physicochemical properties (summarized in Tables S5 and S6) of the A β , insulin and PSM α s. In addition, it will help us verify whether the proposed solubility diagram (Figure 3) coincides with the procedures available in the literature.

Amyloid beta peptides: A β (1-40) and A β (1-42) contain 40 and 42 amino acids, respectively. A β (1-40) contains only 33% hydrophilic residues, 30% charged amino acids and 40% polar amino acids. In the A β (1-40) peptide, the net charge is negative, so this peptide is acidic and should dissolve in basic solutions. A positive GRAVY value (0.06) and a negative average hydrophilicity value (-0.06) indicate that this is a hydrophobic peptide, and therefore, organic solvents may be useful for its dissolution. For A β (1-42), the additional content of two hydrophobic amino acids (nonpolar) makes this peptide more hydrophobic (GRAVY of 0.20, hydrophilic amino acid content is 31%) and contains fewer charged amino acids (28.6%), making it more difficult to solubilize than A β (1-40). Solubility protocols for these peptides recommend the use of mainly HFIP or bases such as NH₃·H₂O and NaOH, less often acidic conditions, which is in line with the proposed algorithm (Figure 3). Pretreatment of A β with HFIP yields peptide solutions with a homogeneous spherical morphology, with dominant α -helical and random coils and less than 1% β -sheet, thanks to HFIP interference with hydrophobic interactions in A β aggregates, disrupting the existing β -sheet structure and stabilizing the α -helical structure [131]. However, TFA ions cause the peptide solution to adopt a low pH, which facilitates dissolution. However, we must keep in mind that the transition of the solution from an acidic to a physiological condition passes through the A β isoelectric point, which can cause reaggregation [27][24][36]. Although reports in the literature show differences in the *in vitro* behavior of A β (1-40) and A β (1-42) peptides, for instance, A β (1-42) is more cytotoxic and aggregates faster than A β (1-40) [41], the solubilization protocols are similar for both peptides.

Other examples, human and bovine insulin, have the same sequence length but differ slightly in amino acid sequence composition, namely, there are two mutations present in the A chain (Thr to Ala and Ile to Val conversion) and a single amino acid mutation in the B chain (Thr to Ala) (Table S4) [132]. However, the general native structure of both peptides remains almost the same [7]. Swapping Thr for Ala causes a minor change in hydrophobicity - bovine insulin is slightly more hydrophobic than the human version, as indicated, for example, by the GRAVY value (0.20 vs. 0.22, respectively). Amyloid fibrils for human and bovine insulin show some structural differences, despite only slight sequence variations, that are observed in the results of commonly used amyloid investigation methods, for example, aggregation kinetics using ThT [132]. The pI of insulin according to the literature is 5.3 [133] or 5.4 [134], which is consistent with the values obtained with physicochemical calculators

(Table S5). A negative net charge value indicates that it is an acidic peptide, so it should dissolve in basic solvents. A positive GRAVY value in both cases indicates that this peptide is also hydrophobic. Moreover, insulin contains 15.7% charged amino acids, only 29% hydrophilic, and up to more than 60% nonpolar residues. These values indicate that organic solvents may be necessary. Parameter analysis suggests that solubilization may be even more problematic than A β . However, the procedures used by the researchers say otherwise. To dissolve insulin, HCl, or acetic acid to bring the pH of the solution to approximately 2 is mainly used [135][136][137][138][139][140][141][115]. In addition, various types of filters are often used [142][143][144][145]. Literature protocols report that insulin is not soluble at neutral pH and can be dissolved in dilute acetic acid, dilute hydrochloric acid (pH 2-3) or 125 mM NaHCO₃; however, alkaline stock solutions are not recommended since high pH increases the rate of deamidation and aggregation [133]. In conclusion, in the case of insulin, the manufacturer's recommendations are not consistent with the procedures used by researchers.

The phenol-soluble modulin peptides PSM α 1-PSM α 4 consist of 20-22 amino acids, and the sequences are shown in Table S4. PSM α s have a positive net charge and pI at pH>9 (Table S5). The high content of nonpolar amino acids (63-75%), a positive GRAVY value and a negative average hydrophilicity value (Table S6) indicate that these are hydrophobic peptides, so it is likely that organic solvents will be needed to dissolve them. PSM α 4 has the highest GRAVY value (1.70) and is the most hydrophobic of all PSM α s, so it should also show the highest aggregation propensity, which is consistent with the results obtained [146]. However, other studies showed that the highest rate of aggregation possesses PSM α 3 [147][148], and it is the most difficult to dissolve. According to the calculated values (Table S5), the second most hydrophobic of PSM α s is PSM α 1. The physicochemical properties of PSM α 4 and PSM α 1 would explain the ability of both of these peptides to form a thermoresistant β -cross structure [147][146]. PSM α 3 has the lowest GRAVY (0.31), average hydrophilicity (-0.48) and number of nonpolar amino acids (75%). Interestingly, it shows an unusual α -cross structure [147] and is the most cytotoxic after fibrillation [148]. The hydrophobicity of PSMs was also studied by Marinelli et al., who concluded that the properties of PSMs are consistent with their distinct functions and that the spatial distribution of the residues in the different PSM α s contributes to their different amyloid propensity [146]. It is interesting that PSM α 2 does not aggregate, despite its properties similar to other PSM α and the high sequence similarity between PSM α 2 and PSM α 1 [147]. This can be explained by the presence of a discrete number of total charges and pKa, which plays an important role in determining its propensity toward aggregation. This pH-dependent shift in pKa is sensible, given that it would be more favorable to have neutral rather than charged groups buried within the hydrophobic core, and therefore protonation is facilitated by aggregation [147]. The collected solubility protocols (Table S3) recommend using mainly a 1:1 mixture of HFIP and TFA or HFIP and 30% NH₃·H₂O or HFIP alone. Very often, DMSO is also added at a later stage, which is surprising since Met-containing peptides are also susceptible to oxidation [36][91]. Moreover, DMSO can promote aggregation [34]. Although reports in the literature indicate differences in the behavior or

even structure of individual PSM α s [147][148], the protocols for their solubilization are similar, analogous to the A β peptide.

CONCLUSIONS

The aggregation of peptides is a complex and multifactorial phenomenon, and the specific mechanisms of the peptides can vary depending on the peptide under consideration. Understanding the factors that contribute to this process is crucial to mitigate or prevent not only peptide aggregation but also protein aggregation-related diseases and to design therapeutic interventions. In turn, the development of these strategies and drugs requires the ability to solubilize peptides to a monomeric form and to address the problem of aggregation. Apart from the intrinsic factors (i.e., sequence composition, net charge, hydrophobicity) and extrinsic factors (i.e., concentration, pH, temperature), other aspects, such as the obtaining and storage methods, must also be taken into account in the context of aggregation and solubilization. Several different strategies and protocols have been reported to dissolve the peptides, but none of them is a one-size-fits-all method for solubilization of even the same peptide (as we showed in the case of A β , insulin and PSM α s). Take-home message: It is essential not only to comprehensively understand how intrinsic and extrinsic factors influence peptide solubility but also to gain insights into the biological environment of the studied peptides. This knowledge is crucial for designing experiments effectively and developing targeted interventions in the complex landscape of peptide and protein aggregation.

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