Deep Eutectic Solvents: Properties and Protein Interactions

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ABSTRACT

In recent years, green solvents have emerged as an alternative to replace toxic organic solvents, deep eutectic solvents (DESs) is a new type of green solvents which have a great development prospect. They are mixtures of two compounds which have a lower melting point due to their unique physicochemical properties. This kind of new green solvents can be used in many fields because of their low toxicity and become more and more popular. Deep eutectic solvents are formed through hydrogen bonding between the hydrogen bond donor (HBD) and the hydrogen bond acceptor (HBA) and have more advantages than traditional ionic liquids and organic solvents. They have many applications in drug delivery and protein extraction. The main physicochemical properties of them have been studied are phase behaviour, viscosity and density in this paper. Moreover, they can interact with protein through several mechanisms which are hydrogen bonds, hydrophobic interactions, salting out effects and electrostatic forces. These mechanisms influence the structure and function of proteins. Additionally, urea and sarcosine are also useful in DES system. In conclusion, DES is a new generation of green solvents and show a wide application prospect in the field of protein science.

Content

1. Introduction

Deep eutectic solvents (DESs) are mixtures of two or more compounds in a certain molar proportion, which can form a eutectic mixture with a lower melting point through hydrogen bonding (Ferreira and Sarraguça, 2024). The word "eutectic" is from a Greek word meaning "low melting". The first known deep eutectic solvent which is urea and choline chloride mixing at 2:1 ratio found by Abbott, et al. (2003) , they found that the melting point of the mixture at this special ratio is lower than the single component (Dzhavakhyan and Prozhogina, 2023). This discovery marks the official proposal of the concept of deep eutectic solvents.

Pollution is an important problem in chemistry and pharmaceutical fields. The solvents used in industries can cause pollutions so that many research focus on finding a green solvent to reduce the pollutions caused by solvents. Green solvents have fewer negative impacts on the environment than traditional solvents. In recent years many studies tried different green solvents to instead traditional organic solvents. Deep eutectic solvents have emerged as a class of green solvents due to their unique physicochemical properties, including high biodegradability, low toxicity and thermal stability (Khan et al., 2025). Nowadays, people pay more and more attention to the application of DESs like drug discovery and drug delivery. Because of their high tunability and low toxicity, DESs are considered to have great potential for improving drug solubility, permeability and bioavailability (Zainal-Abidin et al., 2019). Therefore, DESs have become a key material in multiple industrial fields such as chemical manufacturing, biotechnology processes, and electrochemical technology.

Proteins are the main participant of life activities, they are also essential nutrients of human bodies. They not only can be used in food industries as an excellent food additive but also can be considered as an important ingredient in the pharmaceutical field. Therefore, the technology of protein extraction, transport and isolation are

important in the fields of biochemistry, medicine and pharmacy industry. DESs can interact with proteins, affecting their structure and function of proteins, and this is useful for the research of protein extraction, transport and isolation (Bowen et al., 2022). Compared with the application of traditional solutions in the protein industry, DESs have advantages like high extraction rate, low toxicity and environmental friendliness.

Since 2003, Abbott, et al. proposed the concept of the DES, studies on DESs and proteins have gradually been conducted. Studies on ‘deep eutectic solvent’ have grown rapidly since 2004, reaching 16332 publications in 2025 (Fig.1 A), publications on ‘deep eutectic solvent and protein’ also rise rapidly from 2015 (Fig.1 B). With the promotion of the concept of green chemistry and the development of biotechnology, it is expected that this research direction will continue to receive high attention in the future and promote the in-depth development of related applications.

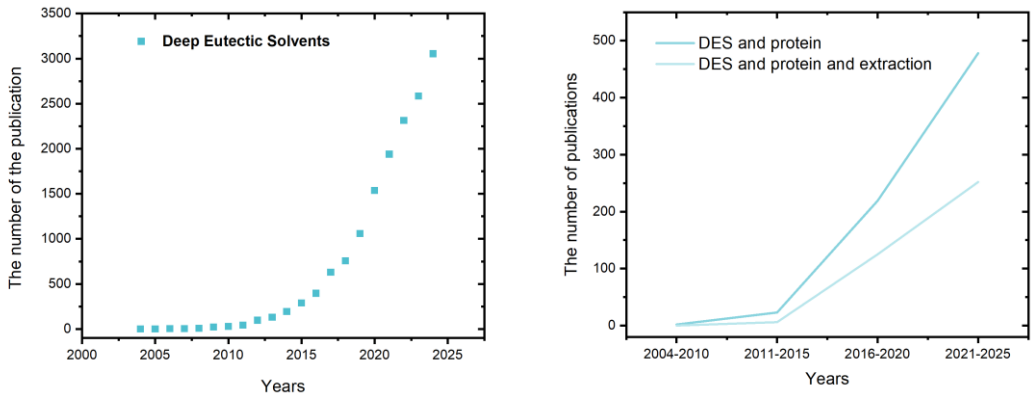

Fig.1 A. Annual publication counts related to the term 'deep eutectic solvents’

B. Number of publications for DES and protein and extraction.

In the field of biomolecular research, the interaction mechanism between DESs and proteins and their applications have become hotspots of current research. This paper reviews the classification, physicochemical properties, and interaction mechanisms with proteins of DESs. Finally, this paper also explains the urea and sarcosine used in DESs.

2. Fundamentals of Deep Eutectic Solvents

2.1 ILs and DESs

Ionic liquids (ILs) have similar properties to DESs which is salt consisting of an organic cation and an inorganic or organic anion (Veríssimo et al., 2024). They are green solvents which were first reported by Paul Walden in 1914 (Sangiorgi et al., 2025) and have been studied for several decades. ILs exist in liquid form at temperatures below 100 °C and have the advantages of low pressure, high viscosity and thermal stability. They were initially regarded as an ideal mixture. In many studies ILs are considered to be excellent solvents to delivery transdermal and oral drugs, as well as good osmotic enhancer (Sangiorgi et al., 2025). Despite the extraordinary advantages and performance of ILs in the extraction process, several studies have raised concerns about ILs due to their anti-biodegradability, high preparation cost, release of harmful secondary metabolites, and time-consuming synthesis process (Chen and Mu, 2021).

The formation of DESs is mainly dependent on strong interactions between hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA). Hydrogen bonds can form a network with van der Waals forces and electrostatic repulsion, which gives DESs specific physicochemical properties such as lower melting points, higher viscosities, higher thermal stabilities and higher solubilities (Ferreira and Sarraguça, 2024). The main difference between DESs and ILs is the presence of hydrogen bonds. Hydrogen bonds network can lead to a lower melting point of solvents than the individual compound in solvents at room temperature (Mr et al., 2021). DESs and ILs have many similar physicochemical properties including low melting point, thermal stability and high solubility (Fig.2).

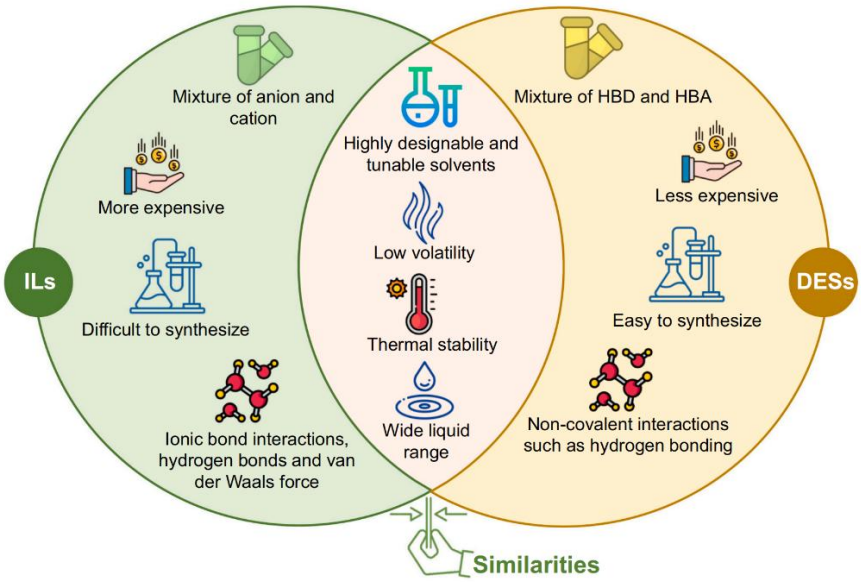

Fig.2 Similarities and differences between ILs and DESs (Veríssimo et al., 2024)

However, compared with ILs, DESs have multiple advantages. DESs are easier to prepare, with high purity, low cost, and no by-products are formed. No purification steps and waste treatment steps are required for DESs. Moreover, they exhibit low toxicity. These characteristics reduce the possible environmental and health impacts (Chen and Mu, 2021).

2.2 Types of DESs

Common HBA are quaternary ammonium salts and metal salts, common HBD are organic acids and alcohols. The properties of DESs can be influenced by different HBA and HBD. In addition to the diversity of HBA and HBD, the molar ratio and purity of HBA and HBD can also affect the physicochemical properties of DESs (Abdelquader et al., 2023). Generally, DESs are classified into five categories by different HBA and HBD (Fig.3).

Four types of DESs were initially set down by Smith (2014). The first type of DESs consists of anhydrous metal chlorides and quaternary ammonium salts which is earliest and the most traditional DES. The second type of DESs is formed by combining

quaternary ammonium salt with a metal chloride hydrate. The third type is composed of quaternary ammonium salts and various organic compounds. This type was discussed most among studies about DESs. The fourth type of DES is synthesised by mixing a metal chloride hydrate with an HBD like urea and ethylene glycol. Emami and Shayanfar (2020) proposed the fifth type, which is environment friendly, is composed of natural amino acids and plant acids such as betaine and citric acid.

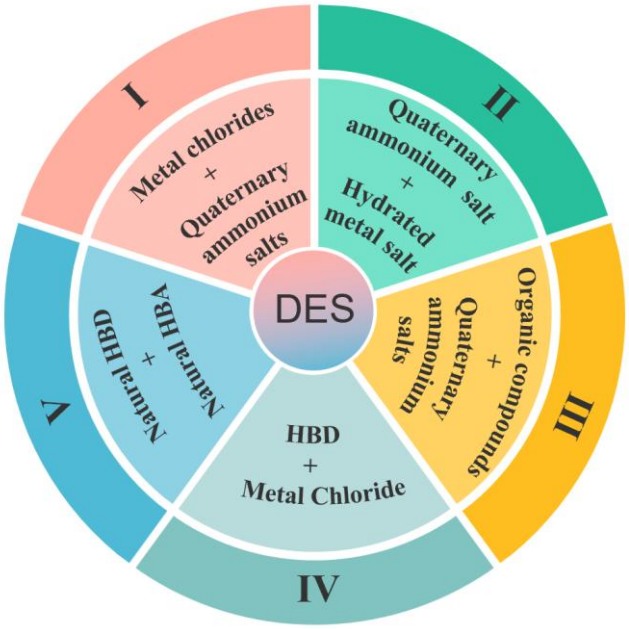

Fig.3 Types of DESs

Natural deep eutectic solvents (NADES) and therapeutic deep eutectic solvents (THEDES) are also two types of DESs which are classified based on the function of DES (Bowen et al., 2022). The main components of NADES are primary plant metabolites such as sugars, acids and amino acids. The concept of NADES is first proposed by Choi et al.(2011) and this kind of DES is usually used for protein extraction. Active pharmaceutical ingredient (API)-based DESs are called therapeutic DES (THEDES) and also can be called API-DES (Abdelquader et al., 2023) which is often used to enhance the dermal or transdermal delivery efficiency of drugs and has a wide range of use in the biopharmaceutical fields. For example, the DES consisting of sulfathiazole and urea is good at antibacterial effects and can regulate the therapeutic effect of drugs (Sekiguchi and Obi, 1961). For the poorly soluble drugs they can provide

an effective method to improve the absorption of drugs (Sekiguchi and Obi, 1961).

2.3 Properties of DESs

DESs have shown broad application prospects in chemistry, medicine, materials and environment fields because of their unique physicochemical properties. Understanding their basic physicochemical properties is important for optimizing its functions and applications. This paper introduces the key physicochemical properties including phase behaviour, density and viscosity of DESs.

2.3.1 Phase Behaviour

DESs are usually a liquid which are clear and viscous. Martins et al. (2019) defined DES as a mixture rather than a compound so that many studies use solid–liquid equilibrium (SLE) phase diagram to evaluation DESs. SLE phase diagram is usually used to describe the phase transition behaviour of binary or multivariate systems (Alhadid et al., 2019). For example, Abbott et al. (2003) study the melting point which is 285.15 K of the DES formed by CHCl : urea in a 1:2 molar ratio. There are two lines in SLE phase diagram. The solid-phase line is a nearly horizontal line, below this line, the system is in a solid state. The liquid line represents the melting point at the different molar ratio of components in a mixture. The intersection point of the solid-phase line and the liquid-phase line is called the eutectic point which is also the minimum point of melting temperatures of the mixture (Fig.4). The main factor affecting the melting point is HBD (Zhang et al., 2012). Many studies have shown that the melting point of most deep eutectic solvents typically ranges from -69 to 149 °C, but they are usually below 150 °C (El Achkar et al., 2021).

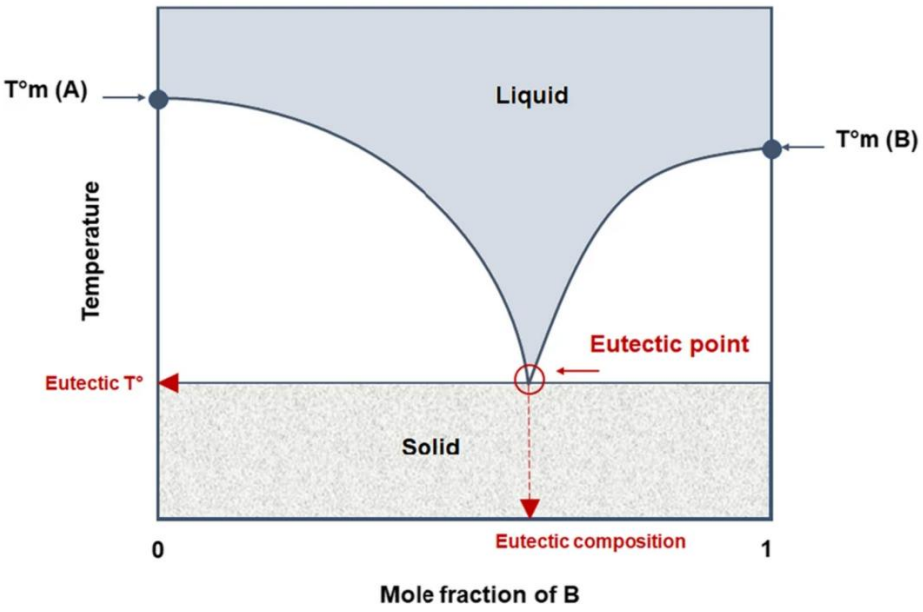

Fig.4 Phase diagram of binary mixture (El Achkar et al., 2021)

2.3.2 Density

Density is a key physical property of solvents. The densities of most DESs are higher than densities of water, which are usually from 1.0 to 1.35 g/cm^3 at 298.15 K (El Achkar et al., 2021). However, hydrophobic DESs have a lower density than the water (El Achkar et al., 2021). The density of a DES is primarily influenced by the combination of HBA and HBD including the type and structure of HBD and HBA (Ijardar et al., 2022). During the formation of DESs, the average pore size are changed when adding the HBD to the HBA, the density of the DES is also changed (García et al., 2015, Ijardar et al., 2022). This phenomenon is also called hole theory. The reason why the DES with the same composition have different densities at different molar ratios can be explained by the hole theory (Zhang et al., 2012). For instance, the density of $ZnCl_2$ - urea at ratio1:3.5 and $ZnCl_2$ - acetamide at ratio 1:4 are 1.63 and 1.36 g/cm^3 respectively (Abbott et al., 2007). Meanwhile, the densities of all the DES decreased with the increase of the studied temperature (Ijardar et al., 2022).

2.3.3 Viscosity

The viscosities of DESs are also one of the most important properties which can affect

the applications of DESs. Most DESs will have higher viscosity than water at room temperature, typically higher than 100cP (Mr et al., 2021). Since each component has a huge hydrogen bonding network, the mobility of free material in DESs is low which leads to high viscosity of DESs (El Achkar et al., 2021, Mr et al., 2021). In addition, other forces like electrostatic or van der Waals interactions in most of the DESs may also lead to high viscosity of DESs (Mr et al., 2021). Also, the water content may alter the hydrogen bonding network of DESs, which may affect its viscosity (Ijardar et al., 2022). According to some studies, the viscosities of DESs may decrease with increasing temperature (Ijardar et al., 2022). Therefore, DESs with high viscosity can be used at higher temperatures. Another example is that the viscosity of the mixture of ChCl and glycerol are different at 20 °C under different molar ratios (Abbott et al., 2011). After the addition of ChCl, the partial break of the hydrogen bond network leads to the reduction of glycerol viscosity (Abbott et al., 2011).

2.3.4 Aqueous DES

Water is one of the most important substances in nature and it is also very important in life and ecosystems. Interactions between DESs and water have special behaviours in physicochemical properties. DESs are usually hygroscopic, the properties of DESs can be changed by the adsorbed moisture in DESs (Palmelund et al., 2021). Aqueous DES was studied by Edler earliest which was defined that DESs are added water into their structures (Hammond and Edler, 2017). After the structures of DESs changing, the density and viscosity of DESs are generally tends to reduce because of the addition of water (Du et al., 2016). This makes them suitable for applications where lower viscosities are required. Aqueous DESs have demonstrated high solubility for various organic and inorganic compounds (Ferreira and Sarraguça, 2024). So, its high solubility makes it suitable for extraction of target compounds especially bioactive compounds and metals. For example, people usually tune the physicochemical properties of DESs through controlling the water content in the food area, especially in the extraction process (Ferreira et al., 2024).

The hydrogen bonding structure of DESs which has a significant impact on the long-term stability of DESs can be changed by the addition of water. As changes on the properties of DESs during long-term storage will influence their reliability and effectiveness in industrial applications, studies on long-term stability are essential to the safe and effective storage of DESs. Jablonský et al. (2018) found that the removal of water molecules under the high temperature may lead to the collapse of the hydrogen bonding network. Therefore, DESs with aqueous components are more suitable for using at moderate temperatures. However, if DESs are added too much water, the hydrogen bond network of DESs may be destroyed and DESs will become an aqueous solution eventually (Dai et al., 2015). At present, although studies demonstrated the potential for tuneable physicochemical properties by controlling the content of water added to DESs (Dai et al., 2015). Current research of aqueous DES is still lacking and require further exploration.

2.4 The Preparation Method of DESs

Mixing HBD and HBA is the main method to prepare DESs, heating and grinding are the two most used methods to mix HBD and HBA. The heating method is based on mixing and heating the HBD and HBA when stirring them until there is a homogeneous liquid formed (Zainal-Abidin et al., 2019). The HBA and HBD are also can be prepared through grinding which is crushing the mixed compounds at room temperature until a clear liquid is formed (Florindo et al., 2014). Some reports have used freeze-drying to prepare DESs by freeze-drying, this method is a type of technique can remove the solvent from the mixing components to prepare DESs (Gutiérrez et al., 2009). There is also another method which is heating the dissolved HBD and HBA in a vacuum condition to evaporate the solvent (Dai et al., 2013). Additionally, for a more rapid preparation of DES, the use of microwave radiation has been proposed by Gomez et al (2018).

2.5 Characterisation of DESs

Characterisation is an effective way to make sure whether the DES is formed as expected. One of unique properties of DESs is tuneable so that DESs can be customised during prepared. Methods of characterisation are main ways to help customise the properties of DESs for applications. Most properties can be characterised by simple physicochemical measurements such as measuring density, pH and viscosity. The melting points can be usually determined through differential scanning calorimetry (DSC), which it is able to measure the melting point and determine whether the DES has formed the expected the eutectic structure (Alhadid et al., 2020). It is also possible to characterise if it is formed the hydrogen bonding network by spectroscopic analysis like flourier-transform infrared spectroscopy (FTIR) (Zainal-Abidin et al., 2019). In addition to hydrogen bond detection methods, different thermal analysis techniques can be used to construct DES phase diagrams and determine their eutectic composition (Zainal-Abidin et al., 2019).

3. DESs and Proteins

Proteins are important biomolecules in living organisms. Most proteins are produced by the original plant and animal parts. With the continuous development of biotechnology, proteins have been widely used in many fields such as medicine, food, materials, agriculture and the environment. Their extraction, purification and functional studies have become the key issues in biochemistry. The applications of DESs in the extraction and isolation of proteins have gradually become a research hotspot. This review provides an overview of the interaction mechanisms between DESs and proteins, their effects on protein, and advanced applications of DESs for protein extraction.

3.1 Mechanism of interaction of DESs with proteins

Proteins are a class of biological macromolecules with complex tertiary structures,

whose conformational stability and functional activity are closely related to the environment. DESs can affect protein behaviour through some mechanisms. The interaction mechanisms between DESs and proteins mainly include hydrogen bonding, electrostatic interaction and hydrophobic interaction.

3.1.1 Hydrogen Bonding

Hydrogen bonding is the main way for DESs to interact with proteins. A stable hydrogen bond network can be formed when hydrogen bond donors and acceptors interact with polar groups on proteins (Fig.5), and then the conformation of proteins is changed (Bowen et al., 2022). In addition, behaviours of proteins in solution are influenced at this time due to the effect between ionic properties of DES and electric charge of proteins (Zhang et al., 2012). Kumoro et al.(2024) pointed that the DES consisting of choline chloride (ChCl) and glycerol are able to form hydrogen bonds with proteins whose solubility and stability will be enhanced. During the extraction of proteins, this hydrogen bonding network helps maintain the activity of the protein so that people can use stable proteins for later applications (Kumoro et al., 2024).

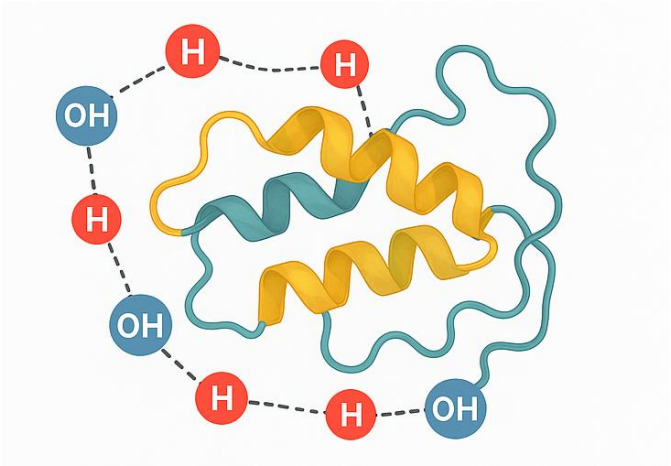

Fig.5 Hydrogen bonding between DESs and proteins

3.1.2 Hydrophobic Interactions

The hydrophobic regions of DESs can interact with the hydrophobic regions of proteins (Fig.6). Bowen et al. (2022) pointed that the tertiary structure of proteins can be

stabilized by this hydrophobic interaction in order to decrease its tendency to aggregate. For instance, the DES composed of ChCl and 1, 4-butanediol can enhance their stability and solubility may because of their hydrophobic interactions (Yue et al., 2021). As this mechanism can significantly improve the extraction efficiency of protein especially when extracting proteins with hydrophobic properties. Research on this mechanism are important for using DESs on protein extraction and drug delivery.

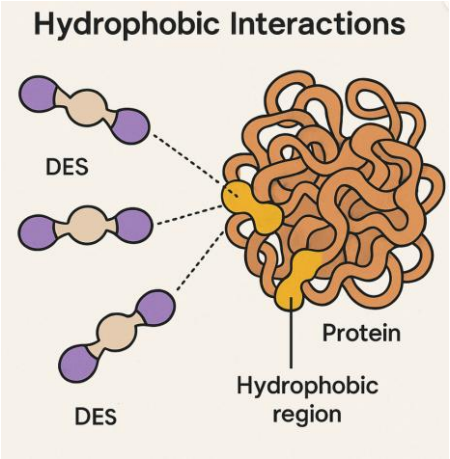

Fig.6 Hydrophobic Interactions between DESs and proteins

3.1.3 Salting Out

The salting out effect of proteins is the phenomenon of reduced solubility and precipitation of proteins at high salt ionic strength. The basic principle of common salting out is that the hydration surface of proteins is destructed, and the charge is neutralized when a high concentration of neutral salt is added to a solution. Proteins will aggregate and salt out because salt ions will combine with water molecules to form hydration ions so that the amount of free water in the solution will reduce (Akagawa and Kudo, 2017). This phenomenon will disrupt the hydration layer around the protein molecules lead to increasing interactions between the proteins and proteins will precipitate. However, in DES system, the salting out effect is not just caused by the addition of salt, when DES interact with salt in protein solutions, there is a ‘salting out-like’ effect on proteins due to the formation of a two-phase aqueous system between DESs and salt to make proteins precipitate (Fig.7) (Bowen et al., 2022). Moreover, protein molecules keep dispersed in solution since there are charges on surfaces of

proteins, and these charges form electrostatic repulsive forces in solution. The charges on the surface of proteins will be neutralized when added salt ions to solutions in order to weak the electrostatic repulsion between the protein molecules, so protein molecules are easier to aggregate and precipitate at this time (Akagawa and Kudo, 2017).

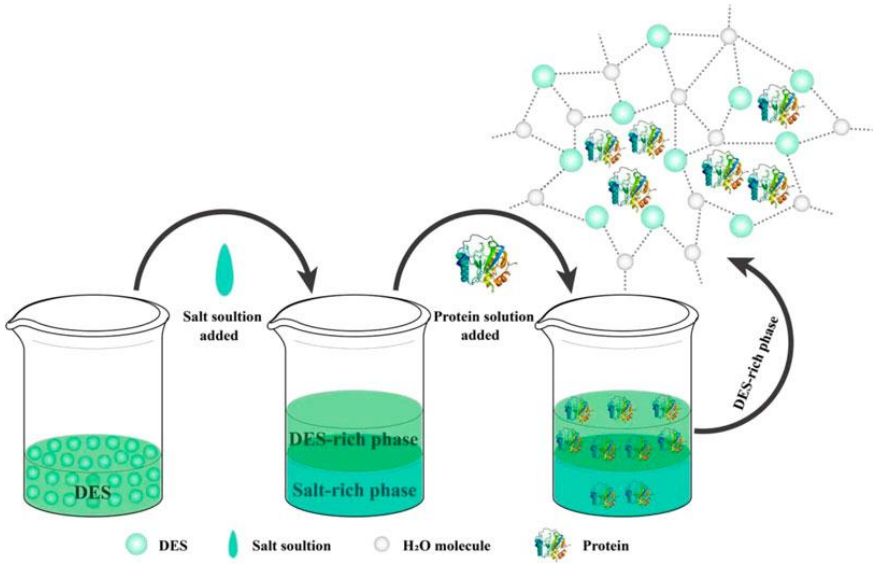

Fig.7 DESs and salt make proteins precipitate (Bowen et al., 2022)

Belviso et al.(2021) found that the interaction of the DES components with specific regions on the surface of proteins affects the solubility of the proteins and then affect the crystallization process. Specifically, when the DES components interact strongly with the protein surface, DESs tend to salt the protein which can also increase solubility, so that crystallization or precipitation can be inhibited. On the other hand, if the DES components tend to interact more with each other and less directly with the protein, protein solubility is essentially unaffected and the conventional salting out process can still proceed normally (Bowen et al., 2022). Xu et al. (2016) mixed choline chloride and glycerol to form ATPS and salt solution, which is used for extracting BSA and he pointed that DES and salt could form a two-phase aqueous system that could be used to enhance the salting out effect.

3.1.4 Electrostatic Force

The ionic nature of DESs allows them to interact electrostatically with proteins. If DESs

are contacted with proteins, the ionic components of DESs can interact with the charge group of proteins and change the charge behaviour and conformation of proteins (Bowen et al., 2022). For instance, some DESs including acidic components like the DES formed by ChCl and lactic acid can be attracted to the amino acid residues which are existing on proteins electrostatically so that enhance the solubilization and extraction efficiency of proteins (Kumoro et al., 2024). When extracting proteins from plant and animal tissues this electrostatic interaction plays an important role because it can enhance the interaction between proteins and solvents and further improve extraction efficiency.

3.2 Effects of DESs on protein structure and function

Proteins are biological macromolecules consisting of amino acids linked by peptide bonds. There are four levels which are primary, secondary, tertiary and quaternary structures of proteins. The biological functions of proteins are influenced by these structures. Proteins are easily influenced by environmental conditions due to their complex structures. Environment changes like temperature, pH, ionic strength, pressure and so on can easily change their structures and make them denature (Veríssimo et al., 2024). Maintaining protein structure and protein stability is good for keep functions of proteins. This is a key issue in the field of biopharmaceuticals.

In recent years, the effect of DESs on proteins structure and function has become one of the research hotspots. Normally, the secondary and tertiary structures of proteins can be affected by hydrogen bonding changing when DESs interact with proteins. But Xu et al (2016) has discovered that certain DESs can extract bovine serum albumin proteins without changing the secondary structure of the protein. On the other hand, the viscosity changing of DESs can also affect the solubility and stability of proteins. It has been shown that some proteins can still maintain high biological activity during the extraction and purification process when some specific DESs are used to protect the activity of proteins (Bowen et al., 2022). In addition, different types of DESs have

different effects on protein folding and structure. Non-ionic DESs have a small effect on the folding and unfolding of proteins, whereas amino acid-based DESs can significantly fold or maintain the natural structure of proteins (Bhattarai, 2024).

For the function of proteins, the enzyme activity is proposed the most which is easily affected by DESs. Some studies found that DESs can enhance the activity of enzymes. For example, the proline-urea DES significantly enhances the activity of trypsin (Taklimi et al., 2023). Increased trypsin activity in DESs can help in the treatment of diseases such as pancreatitis. However, certain DESs which contain urea may reduce the activity of enzymes because the effect of urea on the protein structure (Taklimi et al., 2023). So this function is useful in medicine filed.

Although there are many advantages of DESs in protein research, the research on proteins with DESs is still at the initial stage, they still face some challenges. In addition, issues like biocompatibility and recyclability of DESs are also need to be solved (Bowen et al., 2022). Future research directions may include the development of DESs, the interaction mechanisms between DESs and proteins, and exploration of DESs for biomedical and industrial applications.

3.3 Application of DESs in Protein Extraction

The production of proteins has always been a challenging task. The difficulties people face include extracting proteins from different raw materials (Hanafi et al., 2024). Traditional techniques for protein extraction and isolation include aggregation, salting out, electrophoresis, ion exchange and affinity chromatography. Using DESs is a greener and more protective way than traditional organic solvents for proteins extraction. DESs can not only protect proteins from denaturation but also maintain their natural conformation when extract proteins. For example, choline chloride has shown a protective effect on protein structure in various studies (Khanalipour et al., 2025). There are two types of proteins for extraction which are plant proteins and animal

proteins. The subclasses of plant proteins are albumin, globulin, glutenin, and alcohol-soluble proteins, which are soluble in water, salts, acids, bases, and alcohols respectively (Hanafi et al., 2024). For plants proteins extraction, Chen et al. (2021) found that soy proteins extracted using a mixture of ChCl and glycerol had higher yields and better heat resistance and hydrophobicity than natural soy proteins. The reason why is may be the unique physicochemical properties of DESs. DESs also offer significant advantages in the extraction of proteins of animal origin. For instance, PEG-based DESs can efficiently extract bovine serum haemoglobin up to 88% while maintaining protein stability (Siddiqui et al., 2025).Another advantage for the application of DESs in protein purification is green and environment friendly. Compared with traditional organic solvents, DESs have lower toxicity, higher stability and better recyclability (Kumoro et al., 2024).

DESs have great application potential in the field of protein extraction. It can further enhance the extraction efficiency and purity of proteins by constantly optimizing its components and proportions and contribute to the development of protein extraction production.

4. Urea and Sarcosine in DES

DESs are usually formed by a hydrogen bond donor and a hydrogen bond acceptor at a certain molar ratio. The melting points of them are much lower than the melting points of the single components. Urea and sarcosine are two typical biological small molecules which can impact on stability of proteins, so they have been paid more attention in DESs in recent years. Urea is widely used in DESs studies as a HBD because of its low price and environmental friendliness. Sarcosine (N-methylglycine) is a naturally occurring amino acid derivative containing both carboxyl and secondary amine groups in the molecule. It can be used as not only a HBA but also a HBD. So, combining two gives rise to the new possibility.

Urea as a HBD can combine with other compounds like choline chlorides, amino acids and organic acids to form a stable DES. For example, the mixing choline chloride with urea in a molar ratio of 1:2 can be the DES which has a melting point of only 12℃ (Yu et al., 2022). Urea is non-toxic and biodegradable which is an excellent ingredient of green chemistry. It performs well in extracting natural products and is a widely applicable chemical. Urea-based DESs have good solubility and conductivity and are widely used in metal extraction, electrochemistry and enzyme catalysis (Damjanovi et al., 2024). Urea is a common solvent that can make proteins denature and can interact with proteins through hydrogen bonds and hydrophobic interactions to alter the secondary and tertiary structure of proteins (Wang et al., 2014). Therefore, the stability and folding of proteins are influenced by DESs with urea.

Sarcosine has been studied little in DESs system, but it also has gradually gained attention in recent years. Sarcosine is not only used as a HBA but also participate in hydrogen bond formation due to its carboxyl group. Some studies have shown that sarcosine can form a stable DES with carboxylic acids, cholines, and urea (Damjanovi et al., 2024). In the urea-sarcosine DES, the role of sarcosine is mainly to limit the activity of urea molecules by forming hydrogen bonds with urea which can reduce the denaturation effect of urea on proteins. Damjanovi et al. (2024) found that the DES formed by sarcosine and urea can significantly improve the stability of proteins at a specific molar ratio.

Osmolytes are a type of small molecules that occur naturally in cells and are used to regulate osmotic pressure inside and outside the cell which can maintain protein folding and function. They can affect hydration, protein stability and the structure of biomolecules. Both urea and sarcosine can be regarded as osmolytes, which can affect protein structure. Based on their interactions with macromolecules, osmotic regulators are classified into two groups: ‘kosmotropes’, which stabilize the hydrogen-

bonded structure of water, and ‘chaotropes’, which disrupt intermolecular interactions. Sarcosine belongs to the ‘kosmotropes’ to stabilize proteins, whereas urea belongs to the ‘chaotropes’ and usually destabilizes proteins (Damjanovi et al., 2024). However, at a certain molar ratio, sarcosine can counteract effects of urea on proteins. Damjanovi et al. (2024) reported that urea-sarcosine DESs can still improve the stability of lysozyme after heat shock (80°C) and repeating freeze-thaw cycles (-20°C and -80°C).

The application prospects of urea and sarcosine in DESs are wide. They have demonstrated excellent potential but so far limited to only a couple of examples in the literature. However, the mechanism of action of urea and sarcosine in DESs are not very clear and still need to further explore the interaction of urea and sarcosine these days. Future research can focus on exploring the effects of urea and sarcosine in different DESs systems, as well as their potential in biomedical and industrial applications.

5. Conclusion

Deep eutectic solvents are a type of low eutectic mixtures formed by hydrogen bond donors and hydrogen bond acceptors in a specific molar ratio. They are a new kind of green solvents which are tuneable so that could be used in several applications in varieties of fields. These days, more and more research on them because their low toxicity, high biodegradability and chemical stability. Despite their advantages, DESs also have many unique properties, make them have an important role in protein extraction and purification. They also made a great contribution in improving protein solubility, enhancing stability. When DESs interact with proteins, there are four main mechanisms between DESs and proteins, which are hydrogen bonding, hydrophobic effects, salting-out and electrostatic interactions. The above mechanisms could make DESs to maintain the structure of proteins and reduce the denaturation. Therefore,

DES is an ideal alternative for traditional organic solvents in many fields. In addition, it is also interesting that the combination of urea and sarcosine. Urea is a common component in DESs system, and whereas for the sarcosine, it is not commonly used to make DESs and seldom publications about them in DESs.

Although there are still many challenges in the field of DESs and many unknown problems needed to be further explored. Specifically, due to the short years of research, there is a lack of deep understanding of the DESs system like structures. In the future, the research can focus on the rational design of DESs for specific proteins or specific processes to enhance the understanding of molecular level interactions. DESs will play a key role in the advance of green chemistry and sustainable biotechnology.

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BSA in Urea-Sarcosine DES: Solubility, Aggregation, and Storage Stability

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Abstract

This study investigated the effects of a urea-sarcosine deep eutectic solvents (DES, molar ratio 5:2) at different water contents (50% and 80%) on the solubility, aggregation behaviour, and storage stability of bovine serum albumin (BSA). Results demonstrated that this DES system formed a stable homogeneous liquid, with viscosity and density increasing as water content decreased, while pH remained neutral. At attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) confirmed the formation of a strong hydrogen-bond network in the DES. BSA solubility in the DES increased with rising DES content. Dynamic light scattering (DLS) and analytical ultracentrifugation sedimentation velocity (AUC-SV) revealed that DES effectively inhibited BSA aggregation, demonstrating excellent stability protection particularly under high-temperature (40°C). The 80% water content DES exhibited optimal anti-aggregation efficacy at elevated temperatures. AUC analysis further indicated that 80% water content DES promotes reversible self-association behaviour in BSA, whereas this phenomenon was not observed in 50% water content DES. This study confirms that urea-sarcosine DES is a green solvent which can enhance protein stability, demonstrating potential for application in biotechnology fields.

Content

1. Introduction

Proteins are organic macromolecules composed of amino acids. They possess a quaternary structure, and this structure determines functions of proteins in cells. They can participate in many life processes including enzymatic catalysis, transport, and signal transduction. Studying the structure and function of proteins can help people understand the mechanisms of protein-related diseases and make development for disease prevention and treatment. However, the role of structure makes proteins sensitive to environmental conditions like alterations in pH, temperature, ionic strength and pressure (Dobson, 2003), these can change protein conformation. Only when correctly folded can proteins perform their physiological functions. Once proteins become denatured, physiological functions are impaired, potentially leading to disease. Protein denaturation refers to the disruption of secondary, tertiary and even quaternary structures caused by changes in environmental factors such as temperature, ionic strength and pH. (Kumar, 2009, Pace and Hermans, 1975).

Bovine serum albumin (BSA) is one of the most widely used soluble model proteins in biological research. It is a monomeric globular protein with a molecular weight of 66.5 kDa (Babcock and Brancaleon, 2013). BSA is composed of three homologous domains, each domain is composed of two subdomains, and there are multiple hydrophobic pockets formed internally (Behera et al., 2023). This structure makes the conformation of BSA relatively sensitive to environmental conditions. Because of the stable source, low cost and stable dispersion in aqueous solutions of BSA, it is usually used as a

model to evaluate the effects of solvent environment on protein solubility, aggregation, and conformational stability.

In recent years, because deep eutectic solvents (DES) have been demonstrated to enhance protein stability (Gomes and Galamba, 2023), they have been used in many biotechnologies fields such as protein extraction and separation. DESs represent a class of green solvents formed by hydrogen bond donors (HBD) and hydrogen bond acceptors (HBA) in a certain molar ratio (Ferreira et al., 2024). The melting point of these solvents is lower than that of their individual components due to the presence of hydrogen bond networks in solutions (Dzhavakhyan and Prozhogina, 2023). Compared to traditional organic solvents and ionic liquids, DESs offer advantages such as low-cost materials, straightforward preparation, high tunability and low toxicity (Khan et al., 2025). In addition, the structure and function of proteins can be affected by DESs through some interactions mechanism (Bowen et al., 2022). The addition of water to DESs can alter the hydrogen bond network in mixtures (Bhattacharjee et al., 2024), resulting in changes on viscosity, polarity and density of solvents to change the interaction between proteins and DESs.

Another substance proven to inhibit protein denaturation is osmolytes (Kumar, 2009). They can protect proteins when cells in osmotic pressure changes, salt stress, cold or heat shocks. They through some mechanisms to enhance protein hydration layers and make them stable in in harsh environments (Kumar, 2009). Damjanovi et al. (2024)

proposed DESs can be composed based on osmolyte and this strategy can be used for stabilising proteins under stress conditions like heat shock and freeze-thaw cycles. In their study, urea has been proven to have a stabilising effect on protein, while sarcosine can mitigate the harmful effects of urea on proteins (Damjanovi et al., 2024). On this basis, this study prepares the urea-sarcosine DES with different water content. In this system, urea acts as a HBD to participate in the hydrogen bond network and sarcosine which is a kind of hydrophilic stabilizing agent (kosmotrope) can be used as a HBA in this system.

Based on the above background, this study selects the urea-sarcosine DES to investigate its effects on the solubility, aggregation behaviour and structural stability of BSA under different water content. Analyse the impact of urea-sarcosine DES on BSA through attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), dynamic light scattering (DLS) and analytical ultracentrifugation-sedimentation velocity (AUC-SV) techniques and finally discuss the results.

2. Materials and Methods

2.1 Materials

Bovine Serum Albumin (BSA, catalogue no.1003394773, purity:99%) and urea (catalogue no.00079562, purity:99%) were purchased from Sigma-Aldrich and sarcosine (catalogue no.102446478, purity:98%) was purchased from Thermo Scientific.

2.2 Methods

2.2.1 Deep Eutectic Solvents Preparation

The deep eutectic solvents (DES) were prepared using urea and sarcosine in a 5:2 molar ratio in the method described by Damjanovi et al. (2024). Briefly, to prepare DESs with different water content, urea and sarcosine were added to water up to 50% and 80% (w/w) water content. Samples were heated using a magnetic stirrer at 50°C with 500r/min until form a homogeneous and transparent liquid. Then, solutions remained at room temperature for a week. If there was no solidification or precipitation, DESs were prepared successfully.

2.2.2 Physical Characterisation of DESs

Viscosities were measured by Camlab Ostwald viscometer, 2mL solution was tested in Camlab water bath at 20.0 ºC, assisted by HaakeD8 immersion circulator Schott-Gerate AVS400 automatic pumping & timing. Densities were determined by Auton Paar density meter at 20.0 ºC. The pH of DESs were measured at 23.7 ºC using EasyFive pH meter and calibrated with the three-point method of pH 4.00/7.00/10.00. The above viscosity and density data were used for medium parameter correction in subsequent DLS and AUC.

2.2.3 Solubility of BSA in DESs

The BSA was dissolved in deionised water with concentration from 0.05 to 5.00 mg/mL. These samples were placed in an incubator at room temperature and shaken for 24

hours until complete dissolution, followed by centrifugation for 10 minutes to obtain the supernatant. At 23.0 °C, each concentrate samples were tested absorbance by using a 96-well quartz plate (200μL per well, triplicate wells). Recorded absorbance at 280 nm (Pace et al., 1995) using a TECAN plate reader. Obtain the A_{280} vs concentration standard curve via linear regression. Then added BSA to prepared urea-sarcosine DESs (50% and 80% water content) at an initial concentration of 30 mg/mL. After shaking for 24 hours at room temperature, centrifuged for 10 minutes and collected the supernatant. To ensure absorbance falls within the linear range of the standard curve, the supernatant was diluted to 5 mg/mL. Samples were added into a 96-well plate (200 μL per well, triplicate wells) and absorbance measured at 280 nm. The BSA concentration in DESs was calculated from the standard curve.

2.2.4 Attenuated Total Reflectance Fourier Transform Infrared Spectroscopy (ATR-FTIR)

ATR-FTIR spectroscopy was measured by Agilent Cary 630 FTIR spectrometer equipped with a single-reflection diamond ATR crystal, with data processing conducted via MicroLab Expert software. BSA was dissolved in the DESs to a final concentration of 1 mg/mL. For solution samples measurements, 10-20 μL of sample was added onto the ATR crystal. For solid samples, powder was directly pressed onto the ATR crystal. Before each measurement, background signals were recorded. The scanning range was 4000–650 cm^{-1}, resolution 4 cm^{-1}, with 32 scans per sample at a measurement temperature of 20.0 °C. Data was processed in Origin for baseline correction, Savitzky–Golay smoothing and normalisation, with Y offset during plotting.

2.2.5 Dynamic Light Scattering (DLS)

All DLS measurements were performed using a Zetasizer Nano ZS (Malvern Panalytical, UK). This experiment was conducted at 25.0°C. 1mg/mL BSA was loaded into disposable quartz narrow-wavelength cuvettes at a volume of 100 μL per loading. Following loading, samples equilibrated within the cell for 120 seconds. Each sample underwent three technical replicates under independent loading conditions, and each replicate comprised 10-15 runs. The hydration radius was calculated from the diffusion coefficient via the Stokes–Einstein equation. The different dispersion medium parameters were entered into the software before every measurement (viscosity of 80%water DES: 1.266 cP, viscosity of 50%water DES: 2.568 cP).

2.2.6 Analytical Ultracentrifugation-Sedimentation Velocity (AUC-SV)

Sedimentation velocity experiment was carried out on 2 mg/mL BSA in deionized water, 80% and 50% water content DESs, using a Beckman Optima XL-I analytical ultracentrifuge equipped with Rayleigh interference optics. Samples were run at 45000rpm 20.0 °C in an eight-hole rotor. Both reference and samples channel were added 400μL. Data were analysed by SEDFIT and got the distribution of sedimentation coefficient c(s) (Lebowitz et al., 2002).

3.Results

The urea–sarcosine DESs were prepared at a molar ratio of 5:2 with water contents of

20%,50% and 80%. DESs with water contents of 50% and 80% were transparent and uniform liquids at room temperature (Fig.1). The DES with water content at 20% returned to a solid state after being restored at room temperature (Fig.1). Since the 20% water content DES is not liquid at room temperature, DESs with water contents of 50% and 80% were used in subsequent experiments. During storage for 30 days, no crystallization was observed.

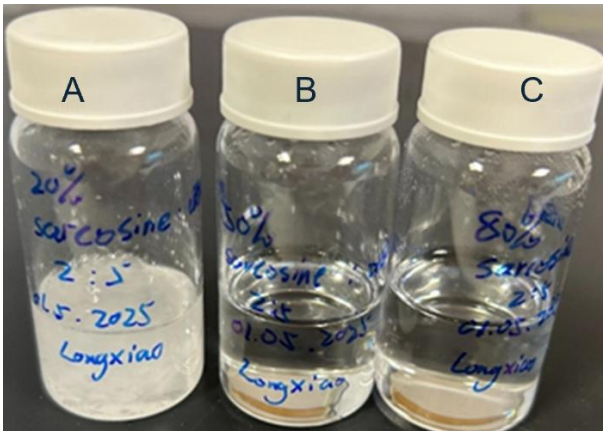


Figure 1. Visual appearance of DESs with water contents of 20%(A),50%(B) and 80%(C)

3.1 Physicochemical Properties

Viscosities (η), densities (ρ) and pH of different water content DESs were tested by corresponding instruments. The results are shown on Table 1. The 80% water content DES viscosity was 1.266 cP and the 50% water content DES was 2.568 cP (Table 1). The results indicate that as the water content decreases, the viscosity of the DES rise. The density of the 80% water content DES was 1.05522 g/cm³, and the 50% water content DES was 1.14392 g/cm³ (Table 1). The density increased with decreasing water content, which may be related to the higher density of the DES components themselves. The pH values of both 80% and 50% water content DESs were 7.01(Table

1), indicating that the addition of DES did not cause significant changes in the acidity or alkalinity of water condition.

Table 1. Viscosities, densities and pH of 80% and 50% water content urea-sarcosine DES

	50% water content DES	80% water content DES
Viscosity (20.0 °C)	2.568 cP	1.266 cP
Density (20.0 °C)	1.14392 g/cm³	1.05522 g/cm³
pH (23.7 °C)	7.01	7.01

3.2 ATR-FTIR

ATR-FTIR is used to analyse the interactions between urea and sarcosine. Figure 2 shows the FTIR spectra of 50% and 80% water content DES and the solid material (received materials) of DES. The overall band shapes of the two DESs are different from the urea and sarcosine, suggesting that intermolecular interactions restructuring after mixing.

Solid urea exhibits a strong and broad N-H stretching near 3350 cm^{-1} (Fig.2). For the aqueous DES, the 3600-3000 cm^{-1} region shows a weak peak characteristic of O-H and N-H vibrations (Biernacki et al., 2020) which is a typical spectroscopic feature of hydrogen bond strengthening. This indicates strengthened hydrogen bonding and the formation of a hydrogen-bonded network in the DES. Both urea and sarcosine contain amide carbonyl groups, show a sharp, intense peak at 1680-1660 cm^{-1} which is C=O (Stuart, 2004) (Fig.2). Compared to the urea and sarcosine, both peaks of DESs have

a red shift and may show slight broadening. This demonstrates that the C=O forms a hydrogen bond so that weaken the signal of the original double bond. In the C-N stretching vibration and N-H bending vibration regions, the solid shows sharp peaks. With decreasing water content of DES, the intensity of these peaks becomes weak and have a slight red shift, indicates the formation of hydrogen bonds in the system.

FTIR results clearly indicate that sarcosine and urea form a hydrogen bond network in the DES, this network causes red shifts and broadening in the N-H and O-H stretching region. The peak position shift in the 50% water content DES is greater than those in the 80% water content DES, further confirming that hydrogen bonding strengthens with increasing DES content.

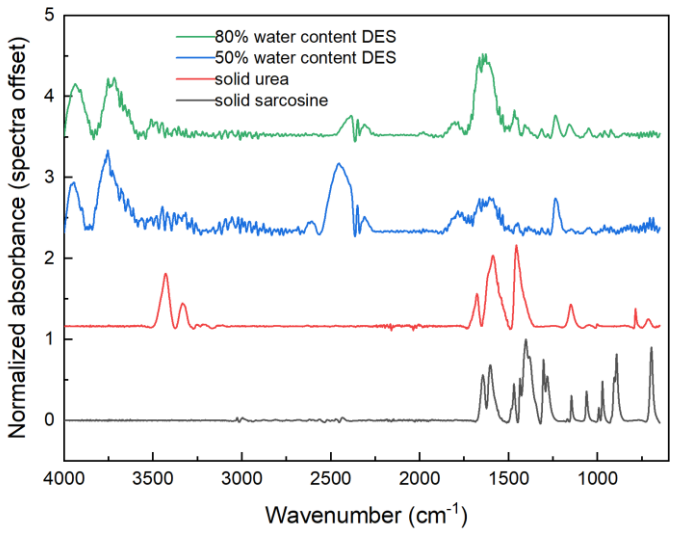


Figure 2. The ATR-FTIR spectra of DES in 50% water and 80% water, with corresponding spectra of urea and sarcosine.

3.3 Solubility

To evaluate the solubility of BSA in the urea–sarcosine DES, this study obtained a

standard curve (Fig.3) of BSA in water. The linear regression equation is:

$$y=0.3572x+0.0694,\ R^2=0.9878$$

Through experiments, the absorbance of BSA in 80% water content DES was measured to be 1.657917 (without background), while in 50% water content DES, the absorbance was 1.838167 (without background). Calculated based on standard curve, the solubility of BSA in 80% water content DES was 4.3260 mg/mL and 4.9518mg/mL in 50% water content DES. Under these conditions, the solubility of 50% water content DES was approximately 14.46% higher than that of 80% water content DES.

Based on the above results, the urea-sarcosine DES can dissolve BSA, and a higher proportion of DES had a slight promoting effect on solubility.

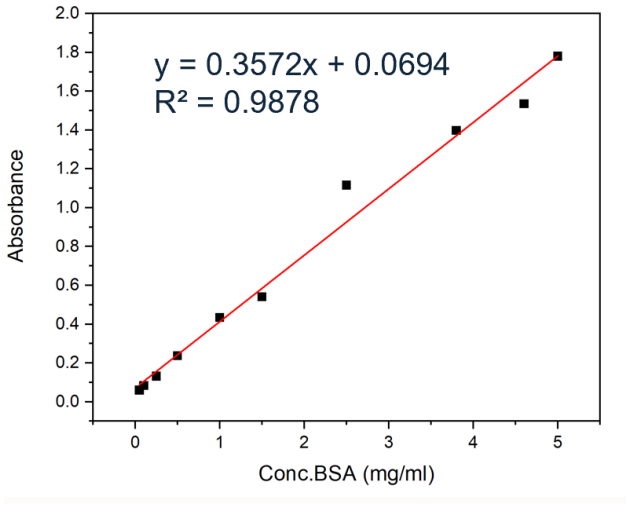


Figure 3. The standard curve of BSA absorbance in water

3.4 DLS

To assess the particle size distribution and aggregation state of BSA in the urea-sarcosine DES, this study measured 1 mg/mL BSA in deionized water,80%and 50%

water content DES using Dynamic light scattering (DLS). Hydrodynamic size was reported as particle mean size and distributions which are shown as volume-weighted figures.

In deionised water, the mean particle size of BSA was 8.070 nm (Table. 2). In 80% water content DES, the mean particle size of BSA was measured was 8.661 nm (Table. 2). Compared to the deionised water, the average particle size increased slightly. In 50% water content DES, the average particle size of BSA was 7.637 nm (Table. 2). Compared to 80% water content DES, the average particle size decreased slightly.

Table 2. Mean particle size of BSA in deionised water, 80% and 50% water content DES

	Mean particle size (nm)
In deionised water	8.070
In 80% water content DES	8.661
In 50% water content DES	7.637

BSA exhibits a unimodal volume distribution in all three systems, but with different peak positions (Fig.4). In deionized water, the main peak is approximately 6–7 nm (Fig.4); in 80% water content DES, it shifts to the right to 8–9 nm (Fig.4); and in 50% water content DES, it shifts further to the right to 9–10 nm (Fig.4). This indicates that in the urea-sarcosine DES, the hydration radius of the BSA increases slightly with lower water content.

The results of the comprehensive volume distribution and average particle size analysis show that the urea-sarcosine DES inhibits the aggregation of the BSA, while slightly increasing the hydration size of the monomer. Overall, the urea-sarcosine DES may improve the stability of BSA, but uniformity still needs further optimisation.

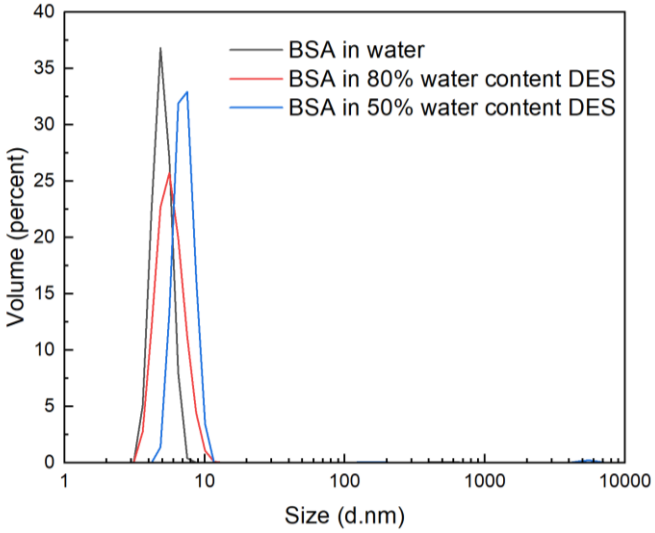


Figure 4. Volume distribution of BSA in water, 80% and 50% water content DES

3.5 Sedimentation velocity

To assess the hydrodynamic properties and aggregation state of BSA in the urea-sarcosine DES, sedimentation velocity was performed with 2 mg/ml BSA in deionized water, 80% and 50% water content DES, and compared the c(s) distribution curves (Fig.5).

In deionized water, the main peak of BSA appears at s_w = 4.398 S and M_w=71.8 kDa, accounting for 85.7%, corresponding to the monomer; a dimer peak is observed at 6.52 S and M_w=129 kDa, accounting for 9.29%. The best-fit friction ratio (f/f0) is 1.42. In 80% water content DES, the monomer peak was at 2.760 S and the dimer at 6.178

S accounting for 7.11% and 9.07% respectively. The result indicates that 80% water content DES may promote reversible self-association of BSA, accompanied by a small amount of protein aggregation. In 50% water content DES, the monomer peak is located at s_w = 4.37 S, and the dimer peak at s_w = 6.39 S, corresponding are 83.9% and 11.3% respectively. The best friction ratio (f/f0) for this sample 0.49.

The c(s) distribution curves show that BSA is predominantly in a compact monomer form in water. In 50% water content DES, it is still predominantly in monomer or dimer form, with only limited self-association observed. In 80% water content DES, the proportion of dimers and small amounts of high-molecular-weight forms increases, suggesting that reversible self-association is promoted.

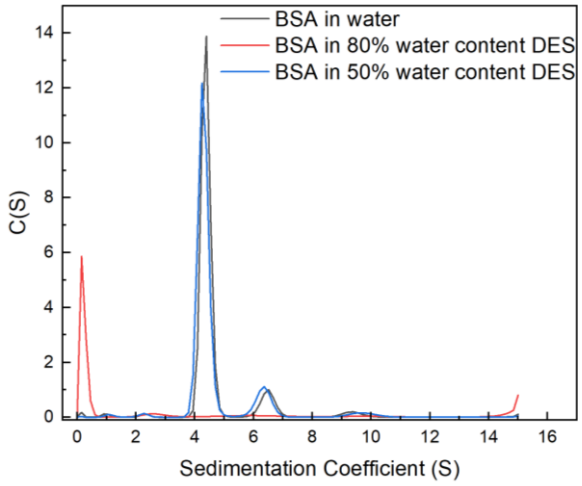


Figure 5. Sedimentation velocity of 2mg/ml BSA in water, 80% and 50% water DES at 20°C.

3.6 Storage Stability

Each sample was stored at -20°C, 4°C, and 40°C for 6 weeks and measured using DLS at 25°C.

According to the mean particle size got from volume distribution data (Fig.6), at 4°C, the size of BSA in deionized water and 80% water DES is close to the monomer size (6-7 nm), but there is an increase in 50% water DES (38 nm). At -20°C, all sizes of BSA slightly Increased (7.1 nm in water, 23.3 nm in 80% water DES and14.4 nm in 50% water DES). At 40°C, there is aggregation occurs, especially in water (950 nm) and 50% water DES (394 nm), while the 80% water DES basically maintains small sized particles (10 nm).

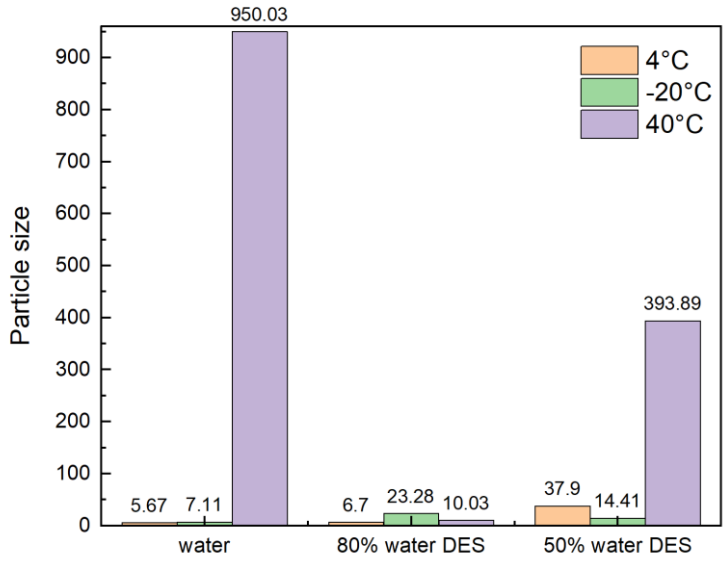
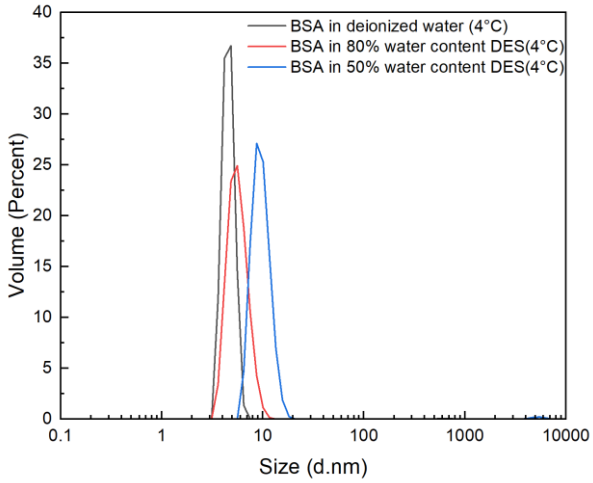


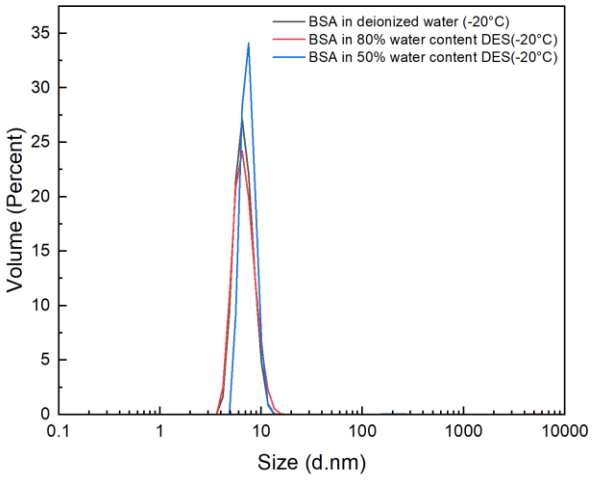
Figure 6. Average particle size of BSA in water, 80% and 50% water content DES at 4°C, -20°C and 40°C (6 weeks)

The volume distribution shows that all three systems are dominated by peaks <20 nm at 4°C (Fig.7 A), with the deionised water peak being the most to the left (approximately 5–7 nm), the 80% water content DES slightly to the right (approximately 7–10 nm), and the 50% water content DES being wider and slightly to the right (approximately 10–30

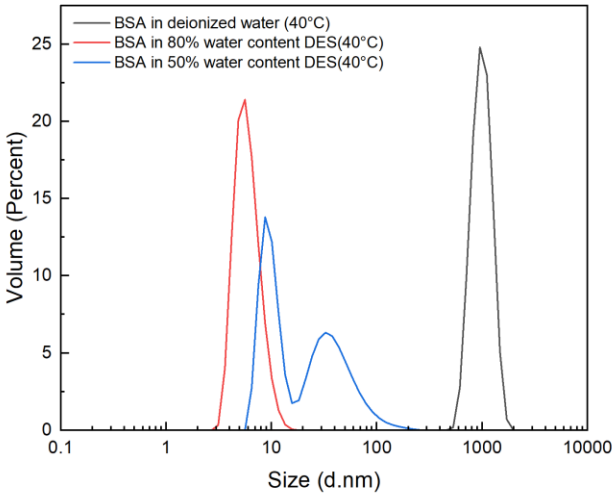
nm). At -20°C, in all three solvents, the volume distribution curve shows a main peak around 6-8 nm (Fig.7 B). At 40°C, the volume distribution shows that the main peak of deionised water shifts to the right to approximately 1μm (Fig.7 C), both DESs maintain peaks within the 10–70 nm range. The 80% water content DES peak is the smallest. The water content 50% DES peak is located at 10 nm, with a slightly wider distribution. The protective effect of DES is most pronounced at high temperatures.



A



B



C

Figure 7 Volume distribution of BSA in water, 80% and 50% water content DES at 4°C (A), -20°C (B) and 40°C (C) (6 weeks)

Combine results of average particle size and volume distribution, DES exhibits anti-aggregation and freeze protection effects on BSA, with 80% water content DES proving particularly effective under high-temperature stress; 50% water content DES is next but still better than pure water. In deionized water, large aggregates are prone to form under high-temperature conditions. Based on the water volume distribution curve, the particle size distribution at -20°C and 4°C is concentrated between 5-8 nm, but at 40°C, the main peak shifts significantly to the right to approximately 1 μm, indicating the formation of large aggregates under high-temperature conditions. In 80% water content DES, the BSA particle size was smaller at 4°C and 40°C, slightly larger at -20°C, indicating that the DES has a protective effect on protein particle size stability at high temperatures, while the water system is prone to aggregation at high temperatures. In 50% water content DES, BSA had the largest particle size at 40°C, which decreased at -20°C. The distribution is relatively monodispersed at -20°C and

4°C, but multiple peaks appear at 40°C, indicating that high temperatures induce the formation of some aggregates.

4.Discussion

This study investigated the effect of urea–sarcosine DES (molar ratio 5:2) on the solubility, aggregation behaviour, and structural stability of BSA. Experiments first confirmed that the stable homogeneous liquid DES was formed with 50% and 80% water content, which the viscosity and density of DES can increase as water content decreases, and pH of both remaining near neutral (approximately 7.0). This demonstrates that DES has higher viscosity and density and good acid-base buffering capacity. Additionally, FTIR analysis showed a noticeable change in the N-H and O-H region, with the hydrogen bond network in the 50% water content DES being denser. This phenomenon is consistent with expectations, the addition of water may cause the formation of a hydrogen-bond network in the DES (Shah and Mjalli, 2014), leading to a reduction in solution viscosity and density.

The research also got the data about solubility, DLS particle size and volume distribution, AUC sedimentation velocity, and six-week storage stability. In terms of solubility, BSA in 50% water content DES was approximately 14.5% higher than that in 80% water content DES, suggesting that a stronger DES network provides a more favourable solvent environment for the outer surface of proteins. However, the structural changes in BSA are not significant in the hydrated DES (Sanchez-Fernandez et al., 2017), so its solubility does not show a marked improvement compared to water.

The DLS results for this experiment exhibited a single peak in all three systems, with the main peak occurring at approximately 6-10 nm. This is consistent with the hydrodynamic radius of BSA monomers reported in the literature (Hawe et al., 2011), indicating that the sample most consists of monomer and has not been structurally disrupted by DES. Compared to deionised water, the main peak shifts slightly to the right at higher DES concentrations. This may because hydrated DES reacting with BSA to thicken the shell or enhance weak reversible association interactions (Fu et al., 2024). In summary, the urea-sarcosine DES did not induce aggregation at this concentration and hydration range but cause a minor effect on the hydration size of BSA.

After storage at -20°C,4°C, and 40°C for 6 weeks, at 4°C, the main peaks of deionised water and 80% water DES still corresponded to the typical hydrodynamic dimensions of BSA monomers. However, the main peak of 50% water DES exhibited a slight rightward shift distribution. At -20°C, the particle sizes increased across all three systems. Freezing during the freeze-thaw process can induce reversible or partially irreversible protein aggregation which is the impact of low-temperature storage (Chi et al., 2003). DES did not change this phenomenon. At 40°C, following long storage aggregated particles formed in water and 50% water DES exhibited hundreds of nanometre peaks with reduced aggregation, whereas 80% water DES maintained peaks within the 10-70 nm range. This demonstrate that the urea-sarcosine DES can protect BSA from thermal aggregation and moderate hydration can inhibit aggregation

under heat environment. Water content is a key regulatory factor, relating to the hydrogen bonds formed between water and DES (Nolasco et al., 2022).

AUC sedimentation velocity results provide a more detailed insight into reversible self-association. The main peak of BSA monomers in water is consistent with classical literature values (Pearson et al., 2015). However, in 80% water content DES, the proportion of dimers increase. The main reason for this phenomenon may be the reversible self-association enhanced (Schuck, 2003). In 50% water content DES, monomers and dimers remain the main components, and the proportion is closer to that in the water. In summary, hydrated DESs can modulate interactions between proteins and the solvation shell, but moderate hydration can enhance protein stability and solubility.

The data discussed forward demonstrate that DES can improve protein solubility and inhibit aggregation under thermal conditions. However, this study also attempted to characterize the conformational changes of BSA in DES using ATR-FTIR and Raman spectroscopy, but under the current conditions (concentration of BSA 1 mg/mL), the signal-to-noise ratio of the protein characteristic peaks was low, and the secondary structure readings could not be obtained. Therefore, these data were not included in the main conclusions. In the future, higher concentrations could be used now that solubility has been determined.

Based on the results of DLS, AUC and storage study, DES generally inhibits aggregation and provides heat and freeze protection within the tested range (-20°C - 40°C), indicating its potential as a protein aggregation regulatory medium. However, 80% water content DES is accompanied by a certain degree of increase in reversible dimers, suggesting that water content can influence the aggregation state, which needs to be balanced and optimized in the future. Overall, this study has demonstrated that urea-sarcosine DES can be an effective green solvent used for regulating protein aggregation. This study provides new data that characterise the physicochemical properties of these formulations and primary data on protein stabilising behaviour. Future work should focus on optimising water and whether any additional formulation excipients (e.g., sucrose) should be incorporated for longer term stability.

5.Conclusion

This study successfully prepared urea-sarcosine deep eutectic solvents with different water contents (50% and 80%). Urea and sarcosine plus water can form a stable homogeneous liquid DES at a 5:2 molar ratio, with FTIR spectroscopy confirming the formation of a strong hydrogen bond network within the DES. As water content decreased, the viscosity and density of the DES increase, while the pH remained unchanged.

The solubility of BSA in the DES increased with rising DES content. DLS and AUC results indicated that the DES effectively inhibited BSA aggregation compared to

deionised water. 6-week stability studies demonstrated that DESs effectively suppressed BSA aggregation under high-temperature (40°C), low-temperature (-20°C) and refrigerated (4°C) conditions, with the 80% water content DES exhibiting optimal protective efficacy at elevated temperatures. AUC sedimentation velocity analysis revealed that the 80% water content DES environment promotes reversible self-association behaviour in BSA, but this did not appear in 50% water content DES condition.

In conclusion, urea- sarcosine DES represents a green solvent which can enhance stability of BSA. It effectively enhances protein solubility and inhibits aggregation through hydrogen bond network formation, demonstrating advantages in countering thermal stress. Future research should focus on investigating the effects of DESs with varying compositions and water contents on different proteins.

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