RESEARCH PAPER

Preparation of Protein/Polymer Nanoparticles for Stabilizing Water in Water Pickering Emulsion

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ABSTRACT

A novel emulsifier based on the conjugation of methoxypolyethylene glycol (m-PEG) with bovine serum albumin (BSA) was synthesized. Lysine was used as the conjugating agent between the protein and the polymer. The nanoparticle was characterized using by FTIR, H-NMR, DLS, and zeta potential analysis. The results confirmed the formation of chemical bonds and a nanoparticle size distribution of 124 nm. The resulting solid nanoparticle as emulsifier was used to stabilize water in water Pickering emulsions consisting a dextran aqueous phase as the continuous phase and a polyethylene glycol aqueous phase as the dispersed phase. Based on the phase diagram of the aqueous polymer solution, water in water emulsions were prepared by adding the emulsifier nanoparticles. Electron microscopy and optical microscopy analyses showed that the average droplet size of the emulsion was 20 micrometers. Fluorescence microscopy was used to confirm the emulsion interface and droplet stability, indicating a stability of 6 weeks. The results of this study demonstrate that the conjugation of proteins and polymers can be used to prepare nanostructures with improved surface properties, which have promising applications in the preparation of new and stable emulsions.

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INTRODUCTION

Many pharmaceutical, food, and cosmetic formulations require the stability of two water-soluble components to prevent the formation of incompatible and immiscible two-phase mixtures. For example, food products often contain immiscible water-soluble mixtures such as proteins and polysaccharides [1]. Protein-based emulsifiers are highly attractive with regard to the promising potential of emulsions in food and biological applications. [2]. However, due to the extremely low interfacial tension and thick interfacial layer in these types of emulsions, achieving stability using surfactants is challenging [3]. Water in water (W/W) emulsions are interesting colloidal dispersions consisting of two immiscible aqueous phases in thermodynamic equilibrium [4]. In W/W emulsions, droplets of one aqueous phase are dispersed in immiscible aqueous phase which are composed of at least two water-soluble molecules that are incompatible with each other. A suitable example of these systems is an aqueous solution

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of two polymers, dextran and polyethylene glycol (PEG). They form two immiscible aqueous solutions based on their phase diagram at specific compositions and separate into two phases [5]. This biphasic solution can be converted into an emulsion system comprising a dispersed aqueous phase in a continuous aqueous phase by using a suitable emulsifier. Dextran is a polysaccharide synthesized by lactic acid bacteria or their enzymes in the presence of sucrose. Polyethylene glycol is a water-soluble, biocompatible, and non-polar polymer derived from ethylene oxide, with a general structure of $HO\text{-}(CH_2\text{-}CH_2\text{-}O)_n\text{-}H$, representing straight-chain polymers.

Pickering emulsions are stabilized by solid, amphiphilic particulate surfactants [6]. Due to their high surface tension, surfactants are adsorbed at the interface of two immiscible phases. Various colloidal particles with amphiphilic properties have been reported as effective emulsifiers in Pickering emulsification [7, 8]. For the preparation of water in water emulsions, different materials such as protein particles including β-lactoglobulin [9] and whey protein [10] have been used. Bioactive protein emulsifier particles (urease, bovine serum albumin) conjugated to methoxypolyethylene glycol have been reported to be produced by Schiff base synthesis and are used to improve the stability of water in water dextran and polyethylene glycol emulsions. This emulsifier acts as a catalyst at the interface and catalyzes the hydrolysis of urea to ammonium carbonate in water [11].

Water in water emulsions of dextran and polyethylene oxide have been reported by using xanthan as a stabilizer (up to 0.5 wt%), exhibiting a stability of one week. However, the results indicated that at lower concentrations, xanthan migrated to the dextran phase, and the instability of the emulsion was the result of a combination of coalescence, creaming, or sedimentation [12].

The water in water emulsions consisting dextran and polyethylene oxide have been reported by using xanthan as a stabilizer (up to 0.5 wt%), exhibiting a stability of one week. The results demonstrated that at lower concentrations, xanthan migrated to the dextran phase, and the instability of the emulsion was a combination of coalescence, creaming, or sedimentation [12]. In another research, aqueous emulsions of dextran and polyethylene glycol using chitosan colloidal particles have been reported, where sodium tripolyphosphate was used for interfacial attachment of the emulsion droplets, resulting in a stability period of more than 1 year, and used for the fabrication of drug-loaded nanofibers [13].

The combination of protein molecules with polyethylene glycol (PEG), a well-established technique known as PEGylation, can effectively improve the stability and potency of proteins [14]. It is believed that PEG chains, covalently bound to the protein, intertwine through hydrophobic interactions around the protein surface, leading to an increase in the size and molecular weight of the protein conjugate. The conjugation between proteins and polymers are rigid and inefficient bonds. The structure of proteins and polymers must be modified in order to improve the efficiency of the bond. The amino acid lysine due to its multiple functional groups, has been used as a modifier for protein and polymer conjugation. Here, the lysine was used to modify the polymer to prepare solid nanoparticles capable of stabilizing water in water Pickering emulsions. The nanoparticles are designed to have their methoxypolyethylene glycol (mPEG) side in the polyethylene glycol (PEG) aqueous phase and their protein (BSA) side in the dextran (DEX) aqueous phase. The use of mPEG polymer, with only one terminal functional group, minimizes the possibility of cross-linking and leads to improved homogeneity of the conjugates.

MATERIALS AND METHODS

Materials

Poly(ethylene glycol) methyl ether (mPEG, Mn~2000) and dextran (DEX, Mn~70000) $[H(C_6H_{10}O_5)_xOH]$ were purchased from Sigma-Aldrich. Lysine, dicyclohexylcarbodiimide (DCC), N-hydroxysuccinimide (NHS), polyethylene glycol (PEG, Mn~20000), and bovine serum albumin (BSA) were obtained from Merck company. All chemicals were used as received without further purification.

Preparation of mPEG-lysine

An esterification reaction was employed to create a linkage between mPEG and lysine. The hydroxyl group at one terminus of the mPEG polymer chain reacted with the carboxylic acid functional group of lysine under acidic conditions. Initially, lysine (limiting reagent) and mPEG were combined in a molar ratio of 1:2, corresponding to a weight ratio of 0.18 g: 2.4 g. Subsequently, hydrochloric acid (5.1 mL, 3 N) was added to the solution and was refluxed at 60 °C [15].

The resulting yellow oily product was stirred at atmospheric pressure to remove residual water. The obtained mPEG-lys was washed twice with acetonitrile and dried completely in an oven for overnight.

Conjugation of Pickering nanoparticles

The conjugation process involved the formation of an amide bond between the amine group of lysine and the carboxylic acid groups of BSA protein in the presence of a catalyst. Specifically, 1 g of mPEG-lys was dissolved in dimethylformamide (DMF), followed by the addition of 0.1 g of BSA. Subsequently, a stoichiometric amount of the coupling agents (DCC & NHS) were added to the solution which was refluxed at 60 °C for 24 hours. The white precipitate of the product was separated from the solution by high-speed centrifugation and washed with DMF for further purification. The precipitate was washed with DMF and centrifuged again under the same conditions. The nanoparticle product (mPEG-lys-BSA) was dried in an oven to remove residual solvent.

Water in water Pickering emulsification

The aqueous solutions of dextran and polyethylene glycol were selected as two immiscible phases. Pickering emulsification of the two aqueous phases was carried out using solid mPEG-lys-BSA nanoparticles. The concentrations of dextran and PEG were chosen to be 2% w/v and 8% w/v, respectively, in a total emulsion volume of 5 mL. 0.1 g of dextran was dissolved in 1 mL of water. 0.4 g of polyethylene glycol was dissolved in 4 mL of water containing 10 mg of dispersed Pickering nanoparticles as an emulsifier. The two aqueous phases were mixed and stirred at a low speed for 5 minutes to form the water in water emulsion.

The effect of BSA amounts on the water in water emulsion' stability was investigated in order to optimize the synthesis method and enhance the emulsification properties of protein-conjugated Pickering nanoparticles. An aqueous biphasic polymeric system without nanoparticles was considered a control sample. Details of the process parameters are presented in Table 1. All samples were prepared at ambient pressure and temperature to ensure consistent emulsion preparation conditions, After the emulsification process, the samples were placed on a flat surface to allow phase separation. After that each phase was analyzed to characterize the resulting emulsion.

Characterization and instrumentation

Fourier transform infrared spectroscopy (FTIR) was conducted using a Magna 550 spectrometer with potassium bromide pellets. Nuclear magnetic resonance spectroscopy (^{1}H-NMR, 410MHz Bruker, Germany) was employed whish heavy water (D_2O) and deuterated dimethyl sulfoxide (DMSO-d6) were used as solvents at room temperature. The particle size and zeta potential of Pickering nanoparticles were analyzed using dynamic light scattering (DLS) with a nano particle analyzer SZ-100. For morphological analysis of emulsion droplets, an Olympus BX53M optical microscope (Japan) and a ZEISS field emission scanning electron microscope (FESEM) were utilized.

RESULTS AND DISCUSSION

Conjugation of m-PEG and BSA

The chemical covalent bonds between proteins and polymers are often characterized by their rigidity and low yield. The polymer structure needs to be modified using small molecule linkers to improve the conjugation efficiency. Lysine was employed as a bifunctional linker to facilitate the conjugation between mPEG and BSA. The synthetic pathway involved an initial conjugation step between mPEG and lysine, followed by a subsequent conjugation step between the lysine-modified mPEG and BSA. A schematic representation of the synthetic route is depicted in Fig. 1.

Spectroscopy spectra of mPEG-lys-BSA

The FTIR spectroscopy was employed to investigate the chemical structure of the products and to confirm the successful completion of the reactions. In Fig. 2 the obtained FTIR spectrum of

Table 1. Characteristics of samples prepared with 1 g of mPEG-lys.

Sample name	Amount (g) of protein
mPEG-lys-BSA1	0.1
mPEG-lys-BSA2	1.0

mPEG-lys-BSA1 was illustrated.

In the mPEG-lys spectrum, the absorption band at 3500 cm^{-1} corresponds to the primary amine of lysine. The absorption band at 1740 cm^{-1} is characteristic of the carbonyl C=O group in the ester bond (RCOOR), confirming the successful reaction. The chemical shift of the carbonyl group of lysine (RCOOH) supports this conclusion.

In the FTIR spectrum of mPEG-lys-BSA, the broad absorption band of the lysine amine group observed at 3500 cm^{-1} in the upper spectrum becomes narrower and shifts to a lower wavenumber (3300 cm^{-1}), which is characteristic of the N-H stretching vibration in amides, indicating the formation of a peptide bond. The absorption band at 1570 cm^{-1} corresponds to the amide bond, characteristic of the N-H bending vibration in the peptide bond. Additionally, the shift of the carbonyl absorption band from 1740 cm^{-1} to a lower wavenumber of 1630 cm^{-1} supports this finding. The appearance of a new absorption band at 1100 cm^{-1}, attributed to the C-N stretching

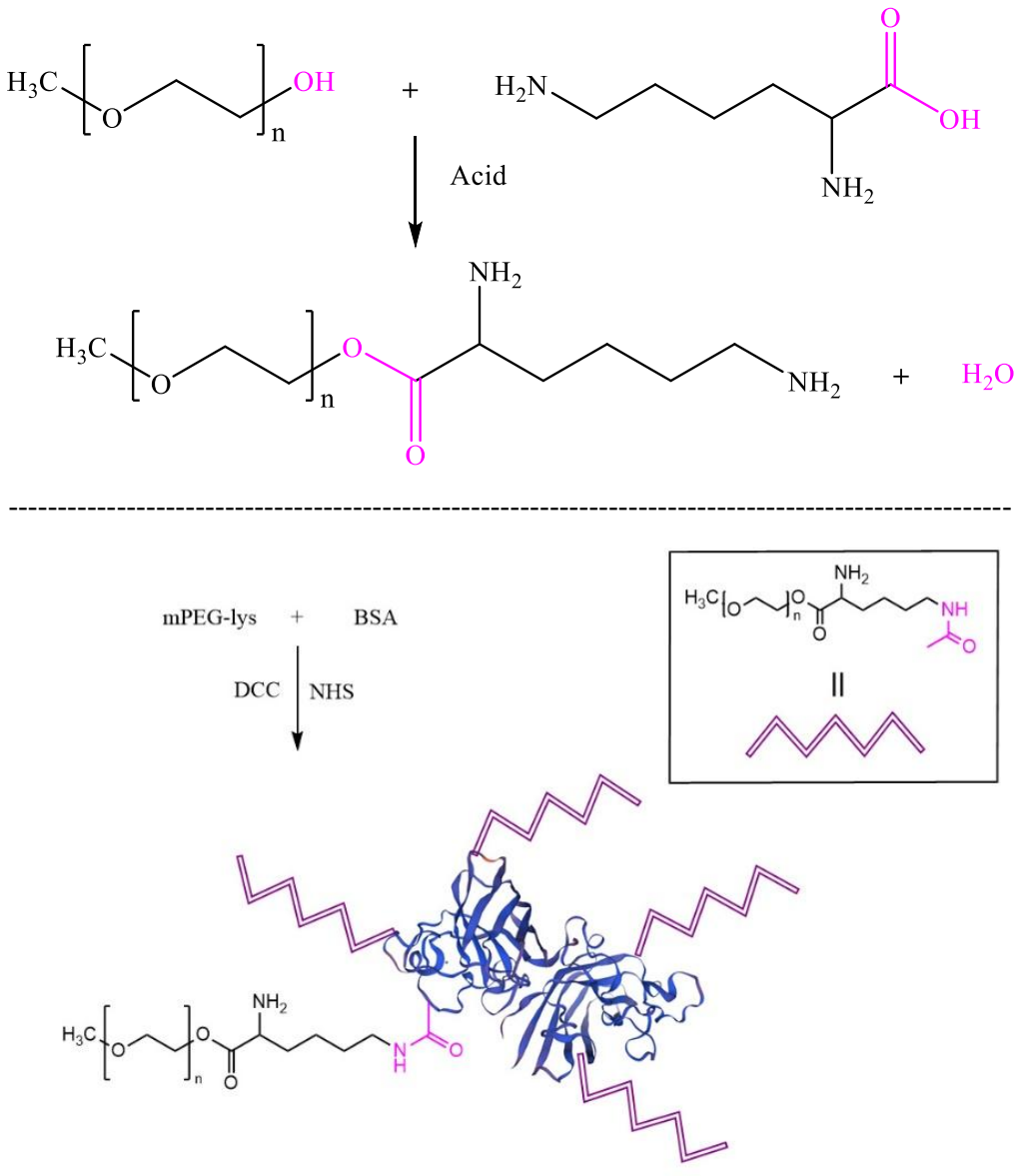

Fig. 1. A schematic representation of the synthetic route m-PEG-lys (upper) and m-PEG-lys-BSA (lower).

vibration in the amide structure, confirms the reaction between the two compounds.

The lysine exhibits a proton resonance signal at a chemical shift (δ) greater than 10 ppm, corresponding to the acidic proton. The mPEG shows a hydroxyl proton resonance signal in the range of 5-7 ppm (broad). As shown in Fig. 3, both of these signals have disappeared, confirming the ester linkage between the two compounds and the formation of mPEG-lys. The other unreacted protons of lys and mPEG are observed in the spectrum of mPEG-lys. The high-intensity peak at 3.7 ppm is attributed to the CH_2 protons in the mPEG polymer chain. The absorbance in the range of 4.6-5.6 ppm correspond to the functional groups in the BSA protein. The peaks between 1.5 and 2 ppm are assigned to the C-H protons in lysine, with the peak at 2.5 ppm corresponding to the C-H bond attached to the NH_2 group. The second peak observed at around 2.6 ppm is attributed to the R(CO)-N-CH protons which are slightly deshielded due to the electronegtivity of the nitrogen atom in the amide bond (R(CO)-N-CH). As a result, the peak appears in the range of 2.2-2.9 ppm. This peak confirms the formation of the amide bond and indicates that the linkage between mPEG-lys and BSA has been successfully established.

Size distribution and zeta potential analysis

Dynamic light scattering and zeta potential analyses were performed to investigate the size and size distribution of the synthesized nanoparticles as well as their surface charge. The results are shown in Fig. 4 and Table 2. The conjugates prepared by self-assembly form colloidal structures in aqueous solutions [16]. The size of these nanoparticles depends on various factors such as temperature, pH, etc. Additionally, ionized functional groups

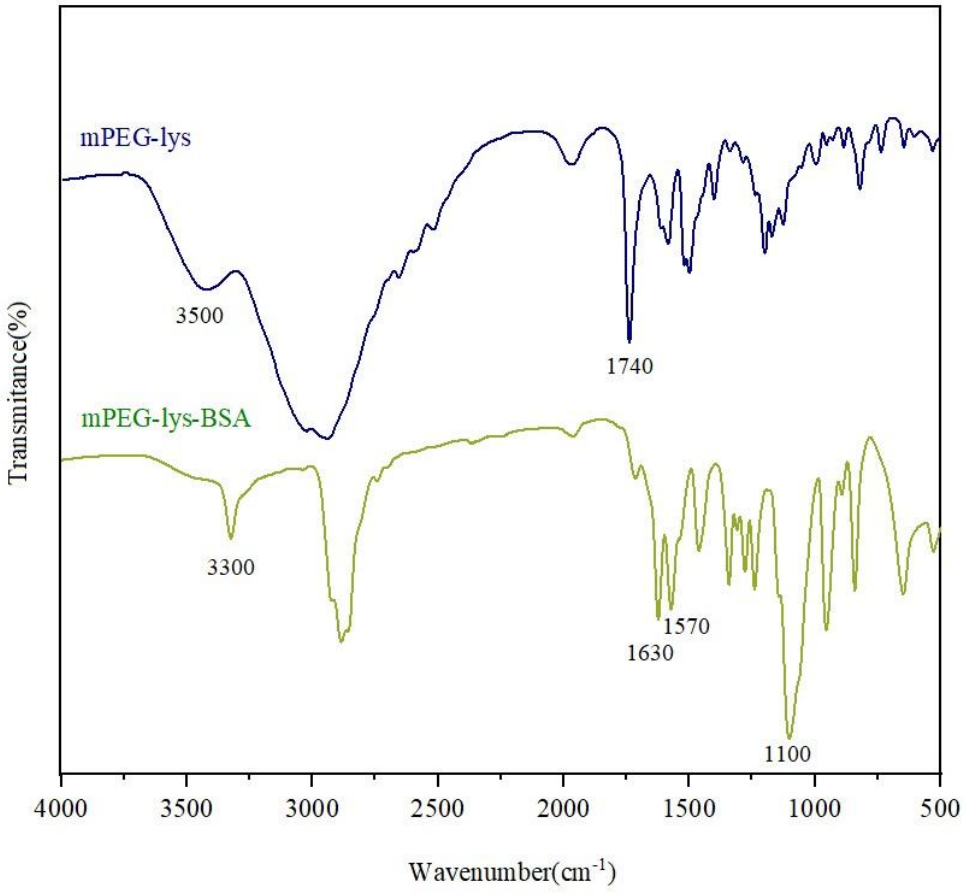

Fig. 2. The FTIR spectra of mPEG-lys (upper) mPEG-lys-BSA1 (lower).

present on the nanoparticle surface give rise to surface charge and zeta potential. As shown in Fig. 4, the self-assembled colloidal particles for both macromolecules exhibit a uniform size distribution. The zeta potential results also indicate a uniform distribution with appropriate values. The size and surface charge of these nanoparticles play a crucial role in the formation of Pickering emulsions and stable droplets.

Formation of W/W Pickering emulsion

Two immiscible polymers were used to create an aqueous two-phase system under specific conditions. Figure 5 illustrates the two-phase solution post-mixing (right side) and following emulsification and phase separation (left side). The phase diagram of the aqueous two-phase system composed of polyethylene glycol and dextran (PEG/DEX) at 22°C is depicted in Fig. 6 [5]. The binodal curve is a crucial feature of the phase diagram, defining the concentrations of components necessary for the formation of either two immiscible aqueous phases (above the curve) or a homogeneous single phase (below the curve).

The percentages of the two polymers for dissolution in water were selected based on this PEG-dextran phase diagram. The predetermined amounts of each polymer (according to the phase diagrams) were dissolved in separate beakers containing distilled water to prepare a water in water emulsion. The nanoparticles (mPEG-lys-BSA) were dispersed in the polyethylene glycol aqueous

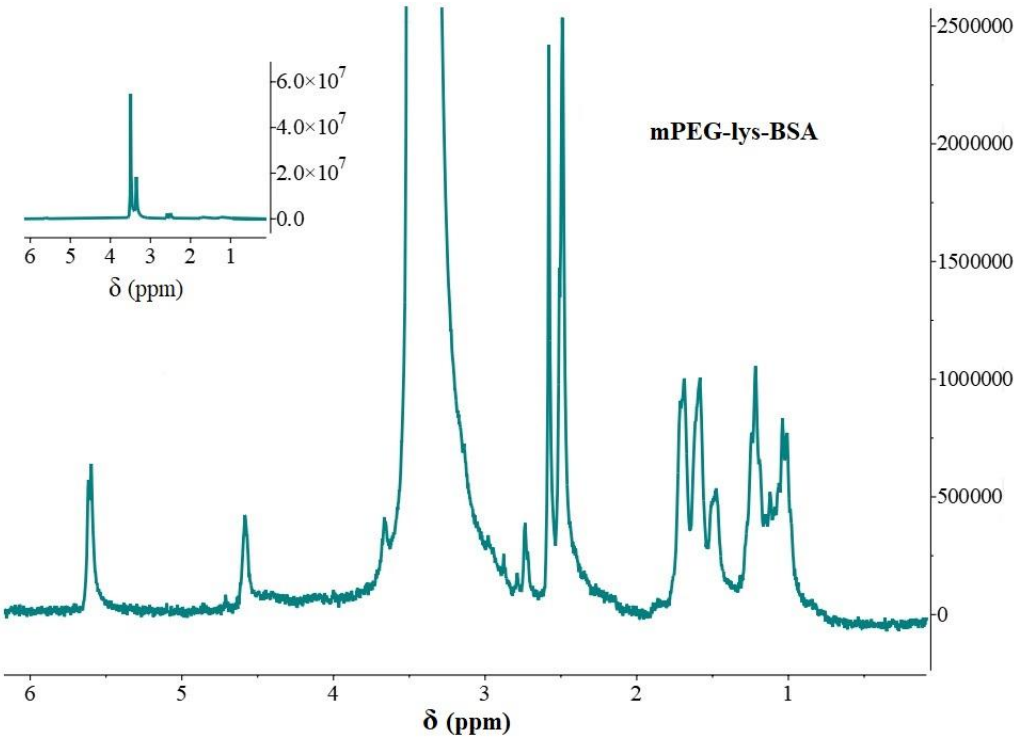

Fig. 3. The ^{1}H-NMR spertrum of mPEG-lys-BSA1 and compressed pattern in zoom.

Table 2. Results of DLS and Zeta Potential Analysis.

sample	particle size (nm)	zeta potential (mV)
BSA1	124	-12

phase and two polymeric solutions were mixed and homogenized using a stirrer (Fig. 5 left). After 50 minutes, phase separation occurred, resulting in a stable water in water Pickering emulsion with PEG (dispersed phase) in DEX (continuous phase) in the lower solution.

Characterization of emulsion

The fluorescence and scanning electron microscopy were employed to analyze the droplet size within the emulsion. The water in water emulsion (PEG in DEX) stabilized using mPEG-lys-BSA nanoparticles (sample BSA1) was directly observed under the microscope after reaching a stable state (Fig. 7). The scale bar in the fluorescence microscopy image is 50 micrometers. The upper image is a bright-field shot of the droplets, while the lower image is the same shot under fluorescence, indicating that the droplets are all approximately 10 to 40 micrometers in size. As observed, the stable spherical droplets remain isolated and separate from each other, and did not coalesce during the sample preparation for imaging or storage. This image reveals the formation of a stable emulsion as it demonstrates a relatively uniform size distribution for the droplets, a

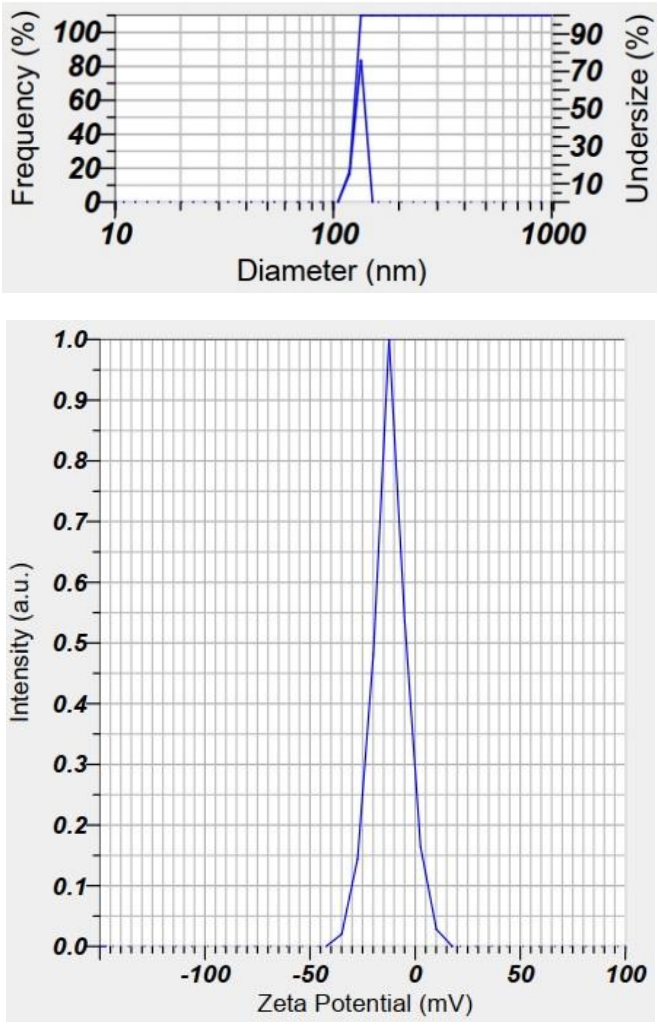

Fig. 4. The size distribution of mPEG-lys-BSA1 (DLS, upper) and surface charge distribution (Zeta, lower).

characteristic feature of stable emulsions in which droplets are well-dispersed.

In order to further confirm the uniformity and size distribution of the droplets, scanning electron

Fig. 5. The illustration of the two phase solution post-mixing (right side) and following emulsification and phase separation (left side).

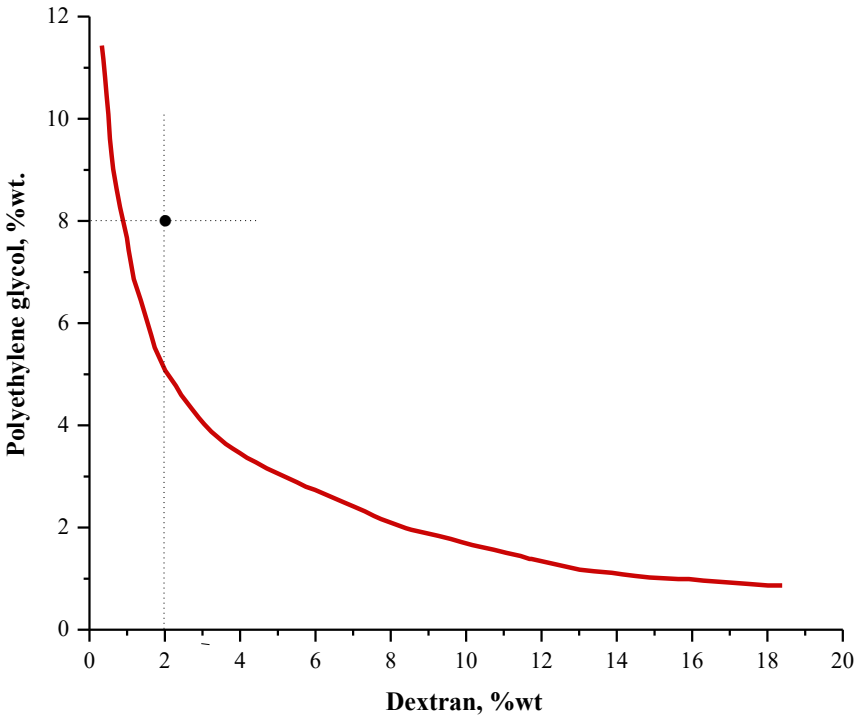

Fig. 6. The two phase diagram of PEG and DEX at 22°C [5].

microscopy (SEM) images are presented in Fig. 7 right. The samples were prepared by depositing a droplet of the emulsion onto a substrate, evaporating the water, and gold sputerring. It is worth noting that this evaporation process led to the collapse of the droplets, reducing their size by up to 50 times. This image reveals a network of discrete spherical droplets with a relatively uniform size distribution of approximately 1 to 2 micrometers. The droplets, exhibiting a consistent size distribution, are dispersed throughout the image, indicating successful emulsion formation that has persisted even after water evaporation.

Emulsion stability analysis

The stability of a prepared water in water Pickering emulsion was evaluated using image analysis. The emulsions were kept stationary for a six weeks period under ambient conditions and then they were imaged. Figure 8 presents the corresponding brightfield and fluorescence microscopy images. The image reveal that mPEG-lys-BSA conjugated nanoparticles were initially well-dispersed in the water in water emulsion, resulting in the formation of spherical emulsion droplets, characteristic of emulsions, and remaining stable for up to 6 weeks.

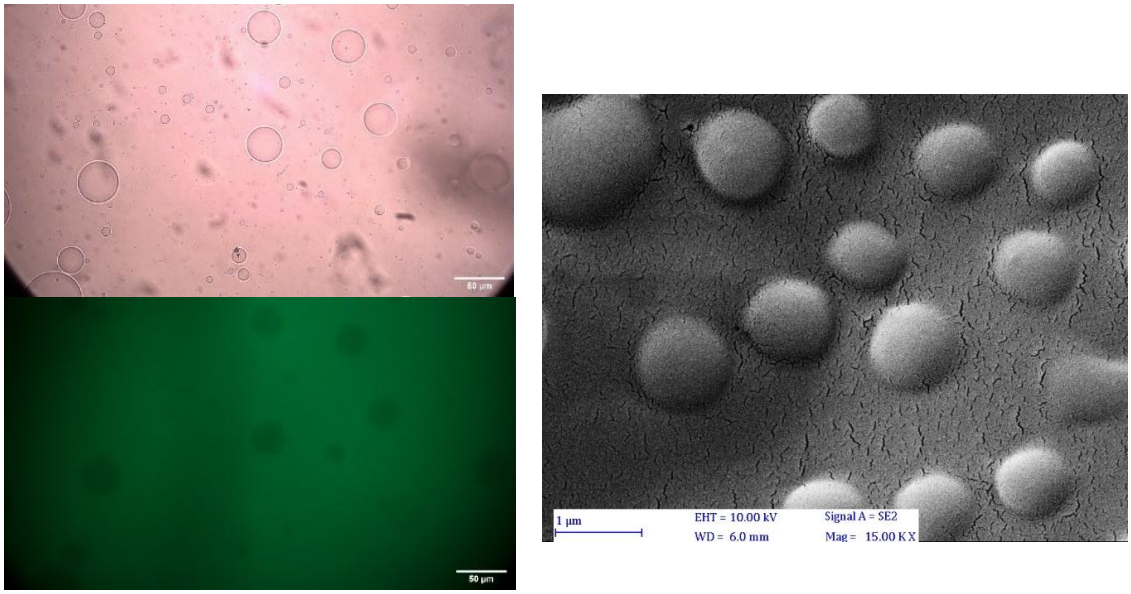

Fig. 7. Left side: corresponding brightfield and fluorescence microscopy images of emulsion droplets, scale bar 50 µm. Right side: uniformity of the emulsion droplets by SEM image.

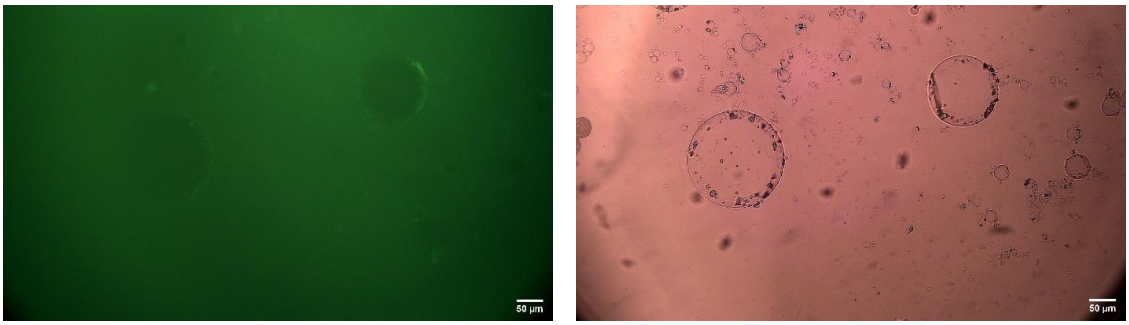

Fig. 8. The representation of stable emulsion after 6 weeks with the corresponding brightfield and fluorescence microscopy images.

The stability of the resulting emulsion droplets can be attributed to the nature of the conjugated nanoparticles. As a result of accumulating at the interface the nanoparticles form a surface layer due to electrostatic forces, thereby stabilizing the size and stability of the emulsion droplets. Furthermore, the zeta potential of the nanoparticles generated an electrostatic repulsion force, which prevented the coalescence of the emulsion droplets, resulting in a more stable emulsion.

CONCLUSION

The formation of water in water emulsions from the phase separation of dextran and PEG polymers is highly challenging due to the extremely low interfacial tension and thick interfacial layer between the two phases. In this research, a solid conjugate of the protein and polymer was used to improve the emulsifying properties of proteins, and its effect on the stability of the water in water Pickering emulsion was investigated. In the preparation process mPEG was initially conjugated with the amino acid lysine, and subsequently, lysine was linked to bovine serum albumin (BSA) protein. The resulting conjugated nanostructure was amphiphilic, with mPEG having affinity for the poly(ethylene glycol) solution phase and the protein BSA molecular chains having affinity for the dextran solution phase. The structural and property analyses confirmed the characteristics of nanostructures. The prepared water in water emulsion had droplets with a diameter of 10 to 40 micrometers, indicating the suitable surface activity of the prepared emulsifier. These emulsifying nanoparticles provided long-term stability of up to 6 weeks for the water in water emulsion, indicating that coalescence of the droplets was prevented. This stability is attributed to the steric hindrance of the polymer chains at the droplet surface and the magnitude of their zeta potential, resulting in the preparation of a highly useful water in water emulsion.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this manuscript.

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