Proteins as Nanocarriers To Regulate Parenteral Delivery of Tramadol

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ABSTRACT: Tramadol (Td) is a centrally acting opioid analgesic drug used for the treatment of moderate to severe pain. However, the half-life of Td is about 6–8 h, which is a major drawback. To increase the half-life of Td, it needs to be entrapped in a suitable substrate with the capability to release the drug for an extended period of time. Accordingly, in our studies, new protein blends in various compositions were prepared using hydrophilic (egg albumin) and hydrophobic (zein) proteins and fabricated them as nanoparticles with Td by the desolvation method. The prepared nanoparticles were characterized using analytical techniques. The morphology and diameter of the nanoparticles were determined by an environmental scanning electron microscope. The interactions between Td and proteins were confirmed by fluorescence spectroscopy, and the secondary structural changes were evaluated by circular dichroism. The hemolysis test and MTT assay indicated that the nanoparticles were nontoxic, and drug release studies showed an extended duration of release of Td for more than 48 h. The mechanism of the drug release followed the zero order. The overall studies inferred that these protein based nanoparticles have potential to release Td at a slow rate for an extended period of time. Further manipulation of the protein composition may regulate the duration of Td release for an effective therapy.

1. INTRODUCTION

In drug delivery, nanomedicine is becoming more popular because the formulations of drugs in the nanoform provide better efficacy at lower concentration and the availability of drug is more at the site of action for the improvement of patient compliance. Accordingly, to design nanomaterials for suitable application, biocompatible, natural, and modified synthetic polymers are being studied extensively.

Proteins are hydrophilic or hydrophobic multifunctional polymers derived from renewable resources and are available in abundance at affordable prices. Therefore, proteins are being extensively studied as drug delivery systems in various forms such as nanoparticles, microparticles, films, and scaffolds. The commonly used proteins are albumin, zein (Ze), collagen, milk casein, soy, whey, and gelatin. Egg albumin (EA) is an abundant protein obtained from egg whites, where the molecular weight is about 40–50 kDa, it is hydrophilic and easily soluble in water, and it has free sulfhydryl (–SH) groups, which on heating form disulfide (S–S) linkages producing stable cross-linked structures. There are few reports on EA for pharmaceutical and biomedical applications and very few reports on drug delivery systems. Besides, Ze is a water insoluble protein obtained from corn and it is recognized as GRAS (generally recognized as safe) by the Food and Drug Administration. Ze being hydrophobic, when dispersed in water it forms aggregates. The aggregates of Ze can be stabilized by increasing the ionic strength or by altering the pH of the dispersing media. Ze showed good biocompatibility as the degraded components of the Ze were easily absorbed and metabolized and therefore being used as micro- and nanoparticles for drug delivery. There are few reports on hydrophobic and hydrophilic drugs immobilized in Ze nanoparticles for slow and controlled drug release. However, hydrophobicity of Ze is one of the limiting factors for its drug delivery application because it inhibits interaction with cells and therefore it cannot be used for invasive delivery of drugs. To expand the scope of this protein, the hydrophobicity of it has to be reduced, which can be manipulated either by functional modification or by physical blending with a hydrophilic polymer. Blending Ze with a hydrophilic polymer would be easy, simple, and economically viable. Ze, modified Ze, and their blends were reported as drug delivery systems. For example, Cheng has stabilized Ze by i-carrageenan, while Wang et al. used sodium caseinate to provide the electrostatic and steric interactions, which prevent Ze nanoparticles from aggregations. In another study Ze...
nanoparticles loaded with 5-fluorouracil were administered via intravenous injection for liver targeting.19 However, new systems using compatible protein combinations are always encouraged in nanomedicine so that the properties of drug delivery will be improved.

Tramadol (Td) is a centrally acting opioid analgesic drug used for the treatment of moderate to severe pain. Among the various opioids used, Td is considered to have low risk of dependence with low abuse potential. Generally, Td is administered orally; however, it has a very short half-life, which is 6–8 h. Increasing the half-life of the drug by immobilizing in a suitable polymer matrix and controlling the rate of release of drug will be an added value in terms of safety and side effects of the drug. Accordingly, there are reports on the application of natural and synthetic polymers as Td carriers for extended release.23–26 The carriers developed for release studies were for oral or parenteral administration, which showed release for 25 h.27–29 Extending the duration of release for more hours by manipulating the properties of the polymers would enable the improved efficacy of the Td application. Therefore, in the present study, we for the first time report the preparation of nanoparticles using EA and Ze, where we could control the hydrophilic and hydrophobic properties to extend the rate of release of Td. The present study involves (i) the preparation of nanoparticles of EA–Ze in various compositions, (ii) analysis of surface morphology and functional and physical properties of nanoparticles by ESEM, FTIR, DSC, and XRD, (iii) interactions of Td with proteins using fluorescence spectroscopy and structural changes by CD, (iv) evaluation of Td release to understand the duration of release, and (v) cell viability and hemolysis studies to identify the toxicity and compatibility of the nanoparticles.

2. RESULTS AND DISCUSSION

2.1. Molecular Weight. Individual solutions of the commercially obtained products of EA and Ze were prepared, precipitated, and lyophilized to obtain pure products. Gel electrophoresis (SDS-PAGE) was used for determining the molecular weights of pure EA and Ze proteins. The molecular weight of EA was 44 kDa, whereas Ze recorded two molecular weights, 22 and 24 kDa. The molecular weights of the respective proteins as determined were in accordance with the reported values30 (Figure S1).

2.2. Nanoparticles and Surface Morphology. The respective nanoparticles of heat cross-linked EA and blends of EA and Ze with and without Td were prepared (Table 1). The desolvation method was used for preparation of nanoparticles according to the procedure as explained in the Materials and Methods section. Proteins, for example, gelatin, collagen, and bovine serum albumin, are generally cross-linked by chemical cross-linkers such as glutaraldehyde, genipin, and carbodiimide to obtain stable materials. However these chemical cross-linkers are considered to be toxic to a certain extent, and one has to be cautious while using them. Therefore, alternative and safe methods are under consideration. EA is abundant with sulfhydryl (–SH) groups, which on heating form irreversible disulfide linkages. Hence, the developed nanoparticles were cross-linked at 50 °C for 4 h, thereby avoiding chemical cross-linkers. The mechanism of heat-cross-linking of EA is given in the Supporting Information (Figure S6).32 We presumed that the nanoparticles of EA, being hydrophilic, will show faster release for short duration. So, to control the rate of release and extend the duration of release, it is desirable to blend the hydrophilic EA with a hydrophobic component. Accordingly, a hydrophobic protein, Ze was chosen to blend with EA. Further, to understand the release kinetics of the drug, the concentration of Td was kept constant at 10% by weight of the total polymer (Table 1) and the blend composition was manipulated by changing the ratios of EA and Ze. In our studies one of the important aspects was to prepare non-aggregates of nanoparticles with uniform size and distribution. Consequently, the particle size analysis data showed the average particle size that ranged between 150 and 650 nm (Figure S5). The polydispersity index ranged from 0.274 to 0.433, and the ζ potential of the EA ranged from −18 to −25 mV, which increased with the addition of Ze at a 1.0:1.0 ratio. However the ζ potential varied from −21 to −23 mV with the addition of 10% Td to the various compositions of nanoparticles of EA and Ze, which may be due to the interactions between positive and negative charges of Td and proteins, respectively (Table S1, Figure S4). The nanoparticles of EA and their blends with and without Td were also examined by ESEM to confirm the particle size, shape, and distribution. The micrographs of the nanoparticles of EA and the blends of EA and Ze with and without drug were analyzed (Figure 1). The cross-linked nanoparticles of EA were spherical in shape, and the average size ranged from 70 to 150 nm in diameter (Figure 1C). The nanoparticles of EA and Ze at a ratio of 1.0:1.0 were also spherical in shape, and the average size of the nanoparticles ranged from 390 to 645 nm in diameter (Figure 1D). EA nanoparticles with 10% Td (w/w) recorded diameters ranging from 309 to 575 nm (Figure 1E).

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Morphology for the compositions C to H (Table 1) of the EA–Ze nanoparticles with and without Td.

**Table 1.** Various Compositions of Blends of Egg Albumin and Zein with Tramadol

| Code | Protein/formulation | tramadol HCl (% w/w of protein) | Size of nanoparticle (nm) |
|------|----------------------|-------------------------------|--------------------------|
| A    | EA (pure)            | 166±12.1                      |                          |
| B    | Ze (pure)            | 632±108.9                     |                          |
| C    | EA heat cross-linked | 10                            | 377.2±29.2               |
| D    | EA–Ze (1:1) heat cross-linked | 10 | 304.6±8.2               |
| E    | EA + drug            | 10                            | 597.3±65.6               |
| F    | EA–Ze (0.5:1) + drug | 10                            | 299.4±11.0               |
| G    | EA–Ze (1:1) + drug   | 10                            | 299.4±11.0               |
| H    | EA–Ze (1:0.5) + drug | 10                            | 299.4±11.0               |
Nanoparticles of the blends of EA and Ze with 10% Td were prepared in various ratios (Table 1). The respective compositions with 10% Td were designated as F, G, and H. The diameters of the nanoparticles of all the different formulations ranged from 300 nm to 1.2 μm in diameter. However, the blends with a 1.0:1.0 ratio showed nanoparticles <500 nm in diameter. The decrease in diameter may be attributed to the increased interactions of Td with EA and Ze (compositions F, G, and H). These interactions were analyzed and confirmed by CD, fluorescence, DSC, and XRD.

2.3. Wide Angle X-ray Diffraction (XRD). X-ray diffraction patterns of EA, Td, Ze, and various nanoparticles were analyzed (Figure 2). The studies were done to understand the changes in crystallinity before and after the formation of nanoparticles. EA did not record any sharp peak as it is amorphous in nature, whereas pure Ze recorded a broad peak at 19° and two sharp peaks at 38 and 45° indicating that it is semicrystalline in nature. The XRD pattern of Td is crystalline in nature. The heat cross-linked EA−Ze blend (D) showed crystalline peaks of Ze at 38 and 45° with reduced intensities due to the functional interactions between EA and Ze. The EA−Ze blend (G) with Td did not show characteristic crystalline peaks of Td, indicating inclusion of Td in the blend and was transformed from crystalline to amorphous due to homogeneous distribution and strong functional interactions between the proteins and Td.

2.4. Differential Scanning Calorimetry. Thermograms of EA, Ze, Td, and their blends were recorded (Figure S2). The thermogram of EA did not record glass transition temperature, whereas Ze recorded $T_g$ at 168 °C, which is close to the reported $T_g$ for Ze (170 °C). The thermogram of EA−Ze blends showed a shift in the glass transition temperature ($T_g$) of Ze from 168 to 171 °C indicating functional and hydrogen ion interactions between EA and Ze. The increase in $T_g$ contributes to the increase in rigidity of the nanoparticles as a result of strong interactions between proteins and Td.

2.5. Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectra of pure EA (A) showed peaks at 3295 cm$^{-1}$ due to $-\text{NH}$ stretching of the secondary amide, $-\text{C=O}$—stretching at 1652 cm$^{-1}$ (amide I), $-\text{NH}$ bending at 1538 cm$^{-1}$ (amide II), and a peak due to $-\text{CH}$ stretching at 2970 cm$^{-1}$. A peak at 1403 cm$^{-1}$ was observed due to plane wagging. The FTIR of cross-linked EA recorded a new peak at 697 cm$^{-1}$ due to disulfide (S−S) bonds, which is attributed to cross-linking of sulphydryl (−SH) groups. FTIR of Td showed well-defined peaks that appeared at 3299 cm$^{-1}$ for the $-\text{OH}$ group, 2945 cm$^{-1}$ for the $=\text{CH}$—of the aromatic ring, and 2843 cm$^{-1}$ for the $-\text{CH}$—stretching. The rest of the peaks of Td were 1595 cm$^{-1}$ for the $-\text{C}=\text{C}$— aromatic stretching, 1464 cm$^{-1}$ for $=\text{C}−\text{H}$—stretching, and 1261 cm$^{-1}$ for $=\text{C}−\text{O}$ stretching. The IR spectra of Ze (B) showed a peak at 3317 cm$^{-1}$ due to $-\text{N}−\text{H}$ stretching, a peak at 2963 cm$^{-1}$ due to $-\text{CH}$ stretching, and the respective peaks at 1664 and 1535 cm$^{-1}$ due to $\text{N}−\text{H}$ and out of plane deformation, respectively. The IR spectra of the nanoparticles of EA−Ze without Td showed the broad peak at 3308 cm$^{-1}$ due to merging of −OH and $-\text{NH}$ of EA and Ze peaks. Similarly the peaks between 2790 and 2930 cm$^{-1}$ of EA and Ze were also merged. Also the characteristic amide I ($-\text{C}=\text{O}$) and amide II peaks ($-\text{NH}$) at 1646 and 1519 cm$^{-1}$, respectively, were merged, which appeared to be broad. The IR spectra of the nanoparticles of EA−Ze blends with Td (G) showed broader peaks, and the corresponding peaks like carbonyl, amine, and hydroxyl groups of protein and Td were very close to each other. Also, we observed some of the peaks disappeared or shifted to a lower or higher frequency. The shifting of peaks indicated hydrogen ion interactions between the protein and Td (for example, 3292 to 3302 cm$^{-1}$, 2970 to 2932 cm$^{-1}$, 1652 to 1659 cm$^{-1}$, 1538 to 1532 cm$^{-1}$) (Figure S3).

2.6. Drug Loading Efficiency, Release Kinetics, and Mechanism. The drug loading efficiency in the nanoparticles was determined using eq 1, where 10 mg of un-cross-linked nanoparticles with Td was allowed to dissolve in 10 mL of dimethyl sulfoxide for ~12 h. The OD (optical density) of the filtered solution containing the dissolved drug was determined at 271 nm. The solution of nanoparticles without Td was used as a reference, and the amount of drug loaded was estimated by the calibration curve of Td. The studies showed that the loading efficiency in all the compositions was about 70 μg.

Figure 2. XRD of EA, Ze Td, and different compositions of EA− Ze nanoparticles with and without Td.
mg\(^{-1}\) (Table S2). The \(\lambda_{\text{max}}\) (271 nm) of Td before and after loading in the nanoparticles was determined to be the same, which was confirmed from our Td release studies. This indicated that Td was stable and active.

Td loaded nanoparticles of various compositions were used for evaluating Td release as a function of time (Table 1). As explained in the Materials and Methods section, the release studies were done in phosphate buffer of pH 7.4 at 37 °C. The release profiles for the compositions of F, G, and H were analyzed. Ten percent (10%) of Td was loaded in the respective compositions F, G, and H. Formulation F showed 18% release within 10 h and 85% release within 48 h; likewise, the formulation G showed 28% release within 10 h and 78% release within 48 h, and the formulation H showed 18% release in 10 h and 77% release within 48 h. It was observed that the release of Td from the composition did not follow any trend (Figure 3). Moreover 'p' values for all the compositions are above 0.1; hence, we conclude that there was no significant difference in the amount of Td released within 48 h.

The drug release profiles of nanoformulations F, G, and H were assessed by zero order, first order, Higuchi, and Korsmeyer−Peppas models. These studies were done to understand the drug release kinetics and mechanisms of different formulations. The drug release kinetics and mechanisms for the respective nanoformulations (Table 1) were analyzed and recorded. Based on the correlation coefficient \((r^2)\), the best fit for the experimental data of release studies was determined. The resultant \(r^2\) values evaluated by various models indicated that the mechanism of release was best fit with zero order. Therefore, we confirmed that the release of Td is independent of the concentration of Td present in the nanoformulations. Further, the Korsmeyer−Peppas release exponent \((n)\) is not uniform for all the formulations, where the formulation F followed anomalous behavior or non-Fickian diffusion, because \(n\) is less than 0.5, indicating the rate of solvent diffusion and polymer chain stretching are comparable. So the release of Td was dependent on diffusion of solvent and chain stretching. Meanwhile, the other two formulations followed a Fickian type of diffusion where \(n < 0.5\), so it indicates that the diffusion of solvent was slower than chain stretching due to increased functional interactions between the drug molecules and the polymer chains of the EA. In these formulations the interactions of Td with EA increased with the increase in EA concentration (refer to CD studies), which is due to the increased affinity of Td with EA compared with Ze (Table S3).

2.7. CD Interaction Study. CD is popularly being used for studying changes in the secondary structure of protein when it gets exposed to various conditions. The CD spectra of the respective proteins EA and Ze were recorded (Figure 4A,B).

The CD spectra for both EA and Ze showed two negative bands that fell between 208 and 222 nm, representing the characteristic feature for the \(\alpha\) helix of the protein. In these studies we also observed that the intensities of the negative bands were increased when Td concentration was increased in the EA solution (Figure 4A). Further, the Ze solution containing low concentration of Td did not record any change in the intensity of the negative band of Td (60 \(\mu\)M). However,
when Td concentration in the Ze solution was increased to 120 and 180 μM, the negative band gradually increased. The increase in the intensity of the negative band indicated a decrease in α helix quantity due to the binding of Td with protein, which is in accordance with the reported literature. From the above studies, we observed that the interactions between EA and Td were more than those between Ze and Td. Therefore, it may be considered that the partition coefficient of Td was more towards EA than Ze.

2.8. Fluorescence Studies for Td Binding with Protein. The binding of drug with protein can be studied by fluorescence spectroscopy. Most of the drug molecules interact with the proteins being multifunctional. CD and fluorescence spectroscopy techniques are widely used for studying the binding interactions between drug and proteins. Egg protein constitutes several proteins in various percentages such as ovalbumin (54%), conalbumin (13%), ovomucoid (11%), lysozyme (3.5%), globulins G2 and G3 (8%), and ovomucin (1.5%). The interactions of Td with EA and Ze were analyzed using fluorescence spectroscopy. The fluorescence spectra for the respective solutions of EA and Ze containing Td at various concentrations were recorded (Figure 5A,B). The intrinsic tryptophan of the EA solution (100 μM) was excited from 260 nm, and we observed an emission at 323 nm. The fluorescence quenching of EA was observed with the addition of Td, which indicated interactions between EA and Td. Nevertheless, the extent of fluorescence quenching remained unchanged with an increase in concentration of Td (4 to 32 μM), because all the available charges (negative) present in EA may have interacted with the positive charges of Td. However, we noticed that the emission wavelength shifted from 323 nm to a lower wavelength, (blue shift, 321 nm), which is due to an increase in hydrophobicity around the fluorophore moiety (for example, tyrosine). The solution of Td alone showed an emission wavelength at 297 nm, which is in accordance with the reported literature. Ze is a hydrophobic protein that is a combination of several polypeptide chains of various molecular weights. In this protein the content of tryptophan is very low and contains a high level of tyrosine. Hence tyrosine was excited to obtain an emission maximum. Ze solution (100 μM) showed an emission maximum at 305 nm, which in the presence of Td at 4 μM concentration recorded appreciable quenching and observed a shift in the wavelength from 305 to 292 nm (blue shift). However with the increase in concentration of Td the quenching was not observed (8 to 32 μM), rather the intensity of the emission wavelength increased without any shift of the wavelength. This indicated that positively charged Td interacts well with EA than Ze, which may be attributed to the hydrophobic nature of Ze.

2.9. Hemolysis Activity. Hemolysis test is one of the methods to show the in vitro biocompatibility of polymeric materials using the RBCs isolated from blood. Ethical clearance was obtained to conduct the hemolysis studies. Accordingly, we obtained blood samples from the volunteers of NCL, Dispensary, Pune, MH, India. As explained in the Materials and Methods section, the negative (phosphate buffer of pH 7.4) and positive controls (deionized water) and the respective compositions of D, E, and G were subjected to the hemolysis test (Figure 6). The results showed 0.7% hemolysis activity for the negative control (phosphate buffer, pH 7.4), whereas the positive control (deionized water) showed 90% hemolysis activity. The nanoparticles without Td of composition D showed 1.20% hemolysis activity, the nanoparticles with Td of composition E showed 0.92% hemolysis activity, and the nanoparticles with Td of composition G recorded 1.09% hemolysis activity (Figure 6A,B). According to the reported literature the materials, which showed hemolysis activity below 5%, are considered to be safe for drug delivery application by the invasive method. From our studies we observed that the nanoparticles of the compositions D, E, and
can consider the developed nanoparticles of EA for drug delivery application. The result was reported in an earlier cytotoxicity study. Thus, we samples as they are not medical grade proteins. Such a type of process of manufacturing the protein) in the pure protein presence of impurities (inseparable impurities during the of samples with respect to the control were between 75 and 25% (Figure 7). The relative cell viabilities recorded for the respective nanoformulation samples (A, B, C, and D) was about 25% (Figure 7). The relative cell viabilities of samples with respect to the control were between 75 and 80%. This difference in cell viability may be attributed to the presence of impurities (inseparable impurities during the process of manufacturing the protein) in the pure protein samples as they are not medical grade proteins. Such a type of result was reported in an earlier cytotoxicity study. Thus, we can consider the developed nanoparticles of EA–Ze–Td for drug delivery application.

3. CONCLUSIONS

Hydrophilic and hydrophobic proteins (EA and Ze) were prepared in different compositions to develop nanoparticles with and without Td by the desolvation method. The size of these spherical nanoparticles of EA–Ze with and without Td was dependent on the composition of Ze and Td. The XRD, DSC, CD, and fluorescence spectra confirmed the binding interactions between proteins and Td. As a result, the crystalline Td became amorphous. Extended duration (48 to 50 h) of Td release (70 to 80%) was achieved from all the compositions when compared with the reported literature on Td release for 24 h. The developed nanoformulations showed initial burst release. The release mechanism of the Td as studied followed a zero order, where the rate of Td release was constant as a function of time. From release kinetics, we conclude that based on the ratios of Ze in the compositions, diffusion of solvent and polymer chain stretching were different and therefore the release exponent (n) was changed. Accordingly the formulation F followed non-Fickian (>0.5) behavior, and G and H formulations followed Fickian behavior (<0.5). The cell viability studies showed an increase in cytotoxicity (2 to 25%) with the increase in EA–Ze nanoparticle concentration (from 62.5 to 1000 μg mL−1). At higher concentration, cytotoxicity was more, which may be due to the presence of possible impurities in the pure proteins. The hemolysis test ranged from 0.92 and 1.20% with and without Td in EA–Ze blends, which indicated that they are biocompatible and therefore may be considered for invasive drug delivery application so as to improve the patient compliance.

4. MATERIALS AND METHODS

Egg albumin powder was purchased from Otto Mumbai, India. Tween 80, non-ionic surfactant (polyoxyethylene sorbitan mono-oleate), and ethanol (absolute) were obtained from SD Fine Chemicals, Mumbai, India. Tramadol hydrochloride was obtained as a gift sample from a pharmaceutical company in India. 3-(4,5-Dimethylthiazol-2-yl)-5-diphenyltetrazolium bromide (MTT), fetal bovine serum (FBS), phosphate-buffered saline (PBS), Dulbecco’s modified Eagle’s medium (DMEM), zein, and trypsin were purchased from Sigma-Aldrich Co., St. Louis, USA. EDTA (ethylenediaminetetra-acetic acid), glucose, and antibiotics were procured from Hi-Media Laboratories Ltd., Mumbai, India. Dimethyl sulfoxide (DMSO), acetone, and propanol were bought from Merck Ltd., Mumbai, India. The 3T3L1 (mouse embryonic fibroblast - adipose like cell line) cell line was procured from the National Centre for Cell Sciences (NCCS), Pune, India.

4.1. Preparation of EA Nanoparticles with and without Td

EA nanoparticles were prepared using the desolvation method. 5% EA solution was prepared in distilled water (w/v). To this solution, 0.5 μL of Tween 80 was added and stirred overnight at 500 rpm. Later, calculated amounts of Td were added to the protein solution and continued stirring for further 2 to 4 h. To this solution while stirring, a desolvating agent, acetone was added (double the volume of Td). To the solution, 0.5 μL of Tween 80 was added and stirred overnight at 500 rpm. Later, calculated amounts of Td were added to the protein solution and continued stirring for further 2 to 4 h. To this solution while stirring, a desolvating agent, acetone was added (double the volume of protein solution) dropwise at a rate of 1 mL min−1. Physically cross-linked nanoparticles were prepared by subjecting the solution at 50 °C for 4 h while stirring at 500 rpm. The obtained EA nanoparticles were purified by two cycles of centrifugation and redispersed in distilled water to remove the

Figure 7. MTT assay for EA (A), Ze (B), EA nanoparticles (c), and EA–Ze nanoparticles (D).
sol fraction. At each centrifugation step, the EA nanoparticles were centrifuged at 15000 rpm for 30 min. After the centrifugation, the pellet of nanoparticles was air-dried. A similar procedure was followed for the preparation of nanoparticles without Td.

4.2. Preparation of Nanoparticles of EA–Ze Blends with and without Td. A similar procedure as described in the above section was followed for the preparation of nanoparticles of the EA–Ze blend. For example, 5% EA solution was prepared in distilled water (w/v). To this solution, 0.5 μL of Tween 80 was added, and the solution of the EA mixture was allowed to stir overnight at 500 rpm. To this mixture, while stirring, 5% Ze solution dissolved in 90% ethanol was added dropwise at a rate of 1 mL min⁻¹. After the addition, the solution mixture was desolvated by dropwise addition of acetone at a rate of 1 mL min⁻¹. The solution mixture of nanoparticles while stirring at 500 rpm was cross-linked by subjecting the solution heating at 50 °C for 4 h. The cross-linked nanoparticles were later purified by two cycles of centrifugation and redispersed in distilled water to remove the sol fraction. At each centrifugation step, the EA–Ze nanoparticles were centrifuged at 15000 rpm for 30 min. After removal of sol fraction the pellet was air-dried and used for further characterization. A similar method was followed for the preparation of nanoparticles with Td where an appropriate amount of Td was added to EA–Ze solution and was allowed to stir for further 2 to 4 h, and later the same procedure was followed as described in the above section.

The Td loading efficiency in the nanoparticles was determined using eq 1:

\[
\text{loading efficiency} = \frac{(\text{total amount of drug added} - \text{free amount of un-entrapped drug})}{\text{the total amount of the drug added}}
\]  

(1)

4.3. SDS-PAGE Electrophoresis. The molecular weights of EA and Ze were determined by SDS-PAGE in a vertical electrophoresis unit (Bio-Rad Laboratories, Hercules, CA, USA). The procedure was based on the reported method with slight modifications. For instance, the sample was denatured by mixing 2 mg of Ze with 1 mL of loading buffer (2.5% SDS, 10 mM Tris HCl, 1 mM EDTA, 6% glycerol, 0.01% bromophenol blue). This sample in the buffer solution was prepared in distilled water (w/v). To this mixture, while stirring, 5% Ze solution dissolved in 90% ethanol was added dropwise at a rate of 1 mL min⁻¹. After the addition, the solution mixture was desolvated by dropwise addition of acetone at a rate of 1 mL min⁻¹. The solution mixture of nanoparticles while stirring at 500 rpm was cross-linked by subjecting the solution heating at 50 °C for 4 h. The cross-linked nanoparticles were later purified by two cycles of centrifugation and redispersed in distilled water to remove the sol fraction. At each centrifugation step, the EA–Ze nanoparticles were centrifuged at 15000 rpm for 30 min. After removal of sol fraction the pellet was air-dried and used for further characterization. A similar method was followed for the preparation of nanoparticles with Td where an appropriate amount of Td was added to EA–Ze solution and was allowed to stir for further 2 to 4 h, and later the same procedure was followed as described in the above section.

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\]  

(1)

4.4. Environmental Scanning Electron Microscopy (ESEM). ESEM (Micro Analysis System and Model Phoenix, Cambridge, England, U.K.) was used for evaluating the morphology of the developed nanoparticles. The samples for ESEM analysis were prepared by cutting a small portion of silicon wafer on which the liquid samples of dispersed nanoparticles were drop-cast and dried. Now the cut portion was carefully mounted on the ESEM stub. The mounted stub was sputtered with gold using an E5000 coating unit (Polaron Equipment Ltd., Watford, Hertfordshire, England, U.K.). A dual beam, having an electron source of tungsten filament (W) with emission at resolution of 20 kV in high vacuum, was used for the studies.

4.5. Polydispersity Index and ζ Potential. The polydispersity index and the surface charge of the nanoparticles were analyzed by a particle size analyzer (90 Plus Particle Size Analyzer, Ver. 3.94, Brookhaven Instruments Corporation, New York, USA). Three milliliters of the sufficiently diluted nanoparticles in distilled water was taken and sonicated for 3 min. The ζ potential of the nanoparticles was measured at 25 °C with a scattering angle of 90°. The polydispersity index was measured to understand the level of homogeneity of particle size.

4.6. Wide Angle X-ray Diffraction (XRD). Crystallographic measurement of the powder samples of polymers and their respective nanoparticles were characterized using a Philips 1830 X-ray diffractometer (Philips, Almelo, The Netherlands). X-rays were generated by a Cu Kα source at a wavelength of 1.54 Å. The samples before and after the formation of nanoparticles were scanned in the 2θ range of 5–45° to investigate the changes in crystallinity.

4.7. Fourier Transform Infrared Spectroscopy (FTIR). Functional characterization of pure samples and blends of nanoparticles such as EA, Ze, Td, and formulations of EA and Ze with and without Td was performed using FTIR (PerkinElmer, Spectrometer I, FTIR diffuse reflectance (DRIFT) mode, USA). The recording of the spectrum was done in the wavelength range of 4000 to 500 cm⁻¹ with a resolution of 4 cm⁻¹. Each spectrum was composed of an average of 12 scans. The respective sample preparation was done by mixing 1–3 mg with ~97 mg of potassium bromide (KBr) and compressed (at 10 ton) to make a pellet, which was then placed in the light path to record the spectrum.

4.8. Differential Scanning Calorimetry (DSC). The thermal properties of the pure polymers, blends of EA–Ze, and Td were investigated by a differential scanning calorimeter (Model Q10 DSC, TA Instruments, New Castle, DE, USA). Overall, 5–6 mg of each sample for analysis was taken in a DSC pan with the lid and sealed it by applying pressure. The sample was equilibrated to ~90 °C for 2 min. In the first cycle, the sample was heated to 200 °C at a rate of 10 °C min⁻¹. In the second cycle, the sample was quenched to ~80 °C at a rate of 100 °C min⁻¹. In the third cycle, the sample was heated to 200 °C at a rate of 5 °C min⁻¹. These experiments were repeated twice for reproducibility.

4.9. Circular Dichroism (CD). The CD studies were done to investigate the effect of the secondary structures of EA (100 μM) and Ze using various concentrations of Td (60, 120, and 180 μM). The CD measurements were carried out in the range of 200–250 nm at 1 nm intervals, and CD spectra were collected with a scan speed of 20 nm min⁻¹. Each CD spectrum was collected with an average of 10 scans.

4.10. Fluorescence Spectroscopy. Fluorescence measurements were performed using an LS50B spectrophluorometer (PerkinElmer, USA). The width of the respective excitation and the emission slits were set at 0.625 nm. The individual solutions of EA with Td dissolved in water and Ze with Td dissolved in 90% ethanol at a concentration of 100 μM EA and Ze, respectively, were prepared, wherein the concentration of Td was varied from 4 to 32 μM. The prepared series of solutions were sonicated (Bio Technics, India) for 10 min, and fluorescence measurements were performed. Fluorescence
emission spectra were recorded in the wavelength range of 260 to 600 nm using an excitation wavelength of 275 nm.

4.11. Cell Line and Subculturing. Stock cells were cultured in DMEM supplemented with 10% inactivated fetal bovine serum (FBS), penicillin (100 IU mL⁻¹), streptomycin (100 μg mL⁻¹), and amphotericin B (5 μg mL⁻¹) in a humidified atmosphere of 5% CO₂ at 37 °C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks, and all the experiments were carried out in 96 microtiter plates (Tarsons India Pvt. Ltd., Kolkata, India).

4.12. Preparation of Test Samples. For the cytotoxicity study, each test sample (polymers/nanoparticles) was weighed, dissolved (polymers) or dispersed (nanoparticles) in distilled DMSO (5%), diluted to the desired volume with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg mL⁻¹ concentration, and later sterilized by UV light.

4.13. Cell Viability Studies by MTT Assay. Cytotoxicity studies for the respective test samples were done by the MTT assay. The monolayer cell culture was trypsinnized, and the cell count was adjusted to 1.0 × 10⁵ cells mL⁻¹ using DMEM containing 10% FBS. To each well of the 96 well microtiter plate, 0.1 mL of the diluted cell suspension (approximately 10000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was discarded, and 50 μL of MTT in PBS was added to each well. The plates were then incubated at 37 °C for 3 days under a 5% CO₂ atmosphere. The microscopic examination was carried out, and observations were noted at an interval of every 24 h. After 72 h, the sample solutions in the wells were discarded and 50 μL of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C under a 5% CO₂ atmosphere. After 24 h, 0.1 mL of the diluted cell suspension (approximately 10000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was discarded, and 50 μL of the diluted cell suspension (approximately 10000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was discarded, and 50 μL of MTT in PBS was added to each well. The plates were then incubated at 37 °C for 3 days under a 5% CO₂ atmosphere. The microscopic examination was carried out, and observations were noted at an interval of every 24 h. After 72 h, the sample solutions in the wells were discarded and 50 μL of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37 °C under a 5% CO₂ atmosphere. The supernatant was removed, 100 μL of propanol was added, and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage of growth inhibition was calculated.

4.14. In Vitro Drug Release Study. The drug release studies were done in phosphate buffer of pH 7.4 in a thermostatically shaking water bath at 37 ± 0.5 °C. To quantify the amount of drug released, the nanoparticles of the respective composition were taken in a dialysis membrane of 1200 molecular weight cutoff (Sigma-Aldrich D-9527) and dialyzed against phosphate buffer of pH 7.4. The sample of nanoparticles without drug was used as a reference. To monitor release of Td, the sample was taken at a regular time interval and the same amount of buffer was replaced. The estimation of the drug was done using the calibration curve of Td. The amount of Td released at each interval was calculated from the OD recorded for each solution at 271 nm using a UV–visible double beam spectrophotometer (Shimadzu UV–1601 PC, Japan). The release studies were monitored up to 48 h in duplicate.

The cumulative percent of drug released(%) = \( \frac{M_t}{M_{∞}} \times 100 \)  

\( (2) \)

where \( M_t \) is the amount of Td released at time \( t \) and \( M_{∞} \) is the total amount of Td present in nanoparticles.

4.15. Evaluation of Percent Hemolytic Activity (HP). Blood was obtained from human volunteers from National Chemical Laboratory, Health Center, Pune, India, and the blood samples were stabilized using EDTA. The red blood cells (RBCs) were isolated from whole blood using density gradient centrifugation. Whole blood of 5 mL was slowly added on top of 5 mL of PBS saline solution and then centrifuged at 2000 rpm for 30 min. The supernatant was discarded, and red blood cells were collected. Later, the RBCs were washed thrice with phosphate buffer saline of pH 7.4 and centrifuged at 2000 rpm for 30 min. A stock solution of RBCs was prepared without serum at 2% (v/v) using phosphate buffer of pH 7.4. Later, 2 mL of the diluted RBC suspension was transferred to each of the 2 mL Eppendorf tubes containing 10 mg of nanoparticles of composition D, nanoparticles of composition E, and nanoparticles of composition G. The negative and positive blood samples were prepared similarly without nanoparticles in phosphate buffer (pH 7.4) and deionized water, respectively. The respective Eppendorf tubes were then incubated for 2 h at 37 °C. During the process of incubation the nanoparticles along with RBCs may settle and, therefore, at an interval of every 30 min, the tubes were shaken gently to resuspend the samples and were centrifuged at 1500g for 10 min at room temperature. The supernatant of PBS was then placed in another 96 well microtiter plate, and hemoglobin (Hb) release was measured spectrophotometrically (OD 550 nm) at 541 nm using a microtiter plate reader (Tecan). The percentage of RBC lysis was calculated based on the assumption that 100% RBC lysis resulted in mixing blood with distilled water at a 1:1 (v/v) ratio. Equation 3 was used for calculating the percent (%) HP.

\[ HP(\%) = \frac{(D_t - D_{nc})}{(D_{pc} - D_{nc})} \times 100 \]  

where \( D_t \) is the absorbance of the test samples and \( D_{pc} \) and \( D_{nc} \) are the absorbances of the positive and negative controls, respectively.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.8b02060.

DSC and FTIR graphs for various compositions, table and figure of ζ potential, particle size analysis and polydispersity index, table of drug loading efficiency, table of drug release mechanisms, table of MTT assay and mechanism of cross-linking of egg albumin by heat treatment (PDF).

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■ ABBREVIATIONS
EA: egg albumin; Ze: zein; Td: tramadol; CD: circular dichroism

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