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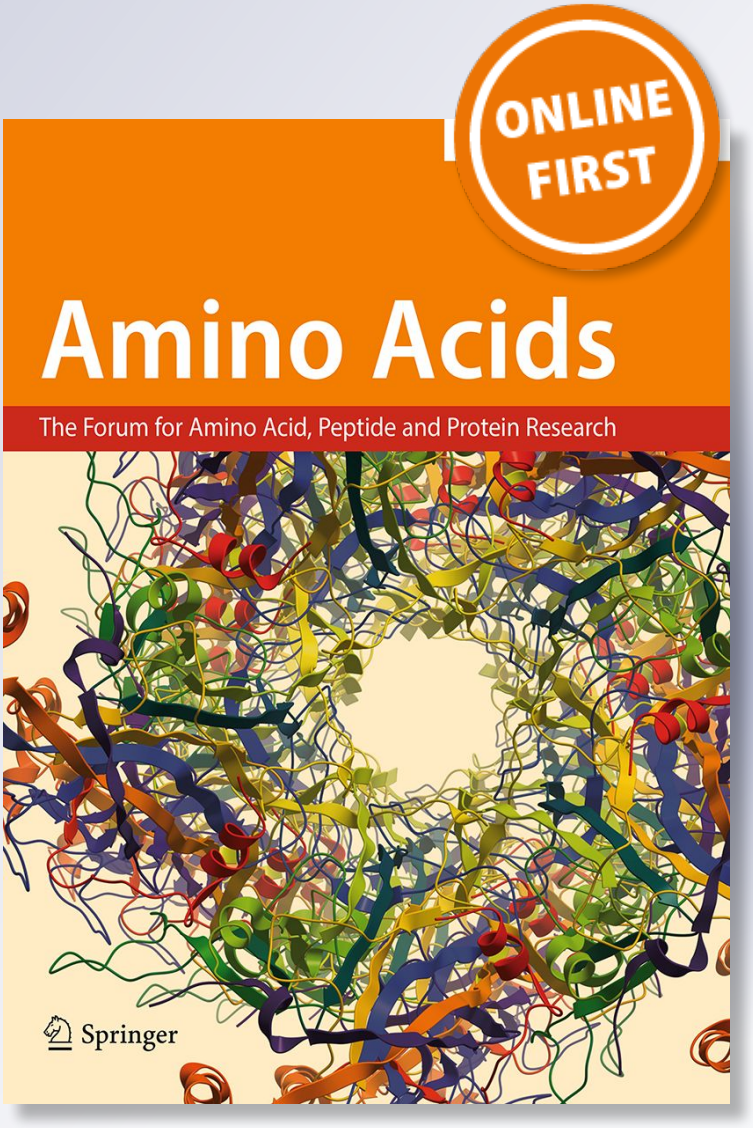

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ORIGINAL ARTICLE

Controlled release of BSA-linked cisplatin through a PepGel self-assembling peptide nanofiber hydrogel scaffold

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Abstract Previously, it has been reported that a novel PepGel (h9e peptide) can be triggered into a solid physical hydrogel by the addition of selected ions and proteins for various biomedical applications. Moreover, PepGel displays shear-thinning and repeatedly reversible sol–gel transfer properties that enable it to be easily transferred via an injector. In this study, PepGel is proposed as a carrier for controlled releases of bovine serum albumin (BSA)-bound or -linked drugs. BSA-linked cisplatin (BSA–CP) is used as a model drug in this study and plays two roles: as a trigger of hydrogel and as a target drug for controlled release. Results of fluorescence instrument show that PepGel significantly quenches the fluorescence of Trp in the hydrophobic subdomain of BSA, indicating a strong interaction. Images of TEM and fluorescence confocal microscopy indicate that BSA–CP is dispersed in the PepGel fibers and at the same time enhances the fiber aggregation. Through UV instrument, it is found that PepGel can effectively inhibit the diffusion of BSA–CP even at concentrations below 0.3 wt% and that the rate of BSA–CP release could be controlled by adjusting the concentration of PepGel. Cell culture studies on the performance of the PepGel are carried out using HeLa cells, and the cell viability is observed to be consistent with the data of drug release. The results showed that PepGel nanofiber scaffolds could potentially be used as an effective carrier for controlled releases of BSA-bound or -linked drugs.

Keywords PepGel · Bovine serum albumin · Controlled release · Peptide

Introduction

Albumin is one of the smallest proteins and the most abundant protein (35–50 g/l serum) present in the blood plasma (Steel et al. 2003). Albumin's three-dimensional binding sites for metabolic substrates as well as diagnostic and therapeutic drugs have been extensively studied and reviewed (Curry et al. 1998; Sulkowska 2002; Kandagal et al. 2006; Agudelo et al. 2012). The protein binds to a great number of therapeutic drugs such as penicillin (Morioka and Tachibana 1995), sulfonamides (Jardetzky and Wade-Jardetzky 1965), indole compounds (Michele et al. 1979), and benzodiazepines (Machicote et al. 2010), to name just a few. Besides, in terms of drug targeting and improving the pharmacokinetic profile of small molecule-, peptide-, or protein-based drugs, albumin is often regarded as a multifunctional protein carrier (Kratz and Elsadek 2012). To be an effective drug carrier, bovine serum albumin (BSA) must provide a well-controlled and sustained delivery method,

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one that can enhance BSA-bound or -linked drugs' pharmacokinetics and pharmacodynamics properties.

Peptide hydrogel provides a potential mechanism of well-controlled and sustained delivery methods. As far as the storage and transfer of proteins is concerned, peptide hydrogel is deemed as a promising material on account of its intensive water content, polymer network, and the capability of maintaining biological activity (Huang et al. 2011). It has long been recognized as well suited for biomedical applications, including 3D cell culture (Huang et al. 2013) and controlled drug releases (Altunbas et al. 2011). In particular, a self-assembling peptide (PepGel) hydrogel system has been developed. It can form a solid physical hydrogel by in vitro assembly at mimic physiological conditions (i.e., neutral pH, room temperature or 37 °C) (Huang et al. 2013). Moreover, PepGel displays shear-thinning and repeatedly reversible sol–gel transfer properties that facilitate the injection and local delivery of drug molecules.

It is potentially desirable to use PepGel for the delivery of BSA-bound or -linked drugs for targeted cancer therapy, in which cisplatin [*cis*-dichlorodiamineplatinum(II)] is one of the widely applied chemotherapeutic agents. Cisplatin is extensively used for treating different types of cancers including testicular cancer, ovarian cancer, bladder cancer, lymphoma, and glioma (Rabik and Dolan 2007). In human blood plasma, the main route for platinum binding lies in the reaction between cisplatin and BSA; and since less than 5% of protein-bound platinum is lost after extensive dialysis, the association is rendered irreversible (Ivanov et al. 1998). In spite of the therapeutic limitations of albumin-bound platinum, some experimental and clinical studies have approved that patient survival span can be significantly increased upon infusion of preformed cisplatin–albumin complexes which are cytotoxic to carcinoma cells (Holding et al. 1992). Besides, some of the side effects of cisplatin treatments might be prevented by albumin binding, in particular its nephrotoxicity (DeSimone et al. 1987).

In this study, BSA–CP was selected as a model drug to determine the effectiveness of PepGel for the stabilization and release of BSA-linked drugs. Previously, it has been discovered that hydrogel from PepGel can be formed when triggered by BSA (Sun et al. 2012). Hence, it is anticipated that PepGel can self-assemble into nanofibers upon interactions with BSA–CP and form a nanofiber-networked hydrogel. To ascertain the distribution of BSA–CP, we imaged the BSA–CP aggregation change with the addition of PepGel solution using TEM and determined the association of BSA–FITC with the PepGel fiber using confocal microscopy. Luminescence was used to detect the interaction between BSA–CP and PepGel, and rheometer was used to detect the gelation of PepGel after mixing with BSA–CP. UV absorbance was performed to measure the release of BSA–CP from PepGel as a function of hydrogel concentration. Furthermore, the anticancer performance of BSA–CP from various concentrations of PepGel was studied in vitro against HeLa cancer cells. This work will enhance the understanding of interactions between BSA and PepGel; it will provide evidence that PepGel could be a potential carrier for the controlled release of BSA-bound or -linked drugs.

Materials and methods

Hydrogel preparation

PepGel with a concentration of 1 wt% h9e peptide was provided by Dr. Sun in the Biomaterials and Technology Laboratory of Department of Grain Sciences, Kansas State University (Manhattan, KS). For hydrogelation, PepGel solution was added into PBS buffer solution (Sigma Chemical, St. Louis, MO, USA) with a final peptide concentration of 0.1, 0.2 or 0.3 wt% and triggered with 1.5 wt% BSA–CP. The hydrogel formed within 15 min at room temperature or 37 °C.

Preparation of BSA–CP complexion

The BSA–CP complex was prepared in a manner similar to that described by DeSimone et al. (1987). Cisplatin (Sigma Chemical, St. Louis, MO, USA) was freshly dissolved in DI water with 100 mM KCl to a concentration of 3 mg/ml in a sterile glass bottle, followed by the addition of freeze-dried BSA to a final concentration of 20 wt%. The molar ratio of cisplatin to albumin in the solution was around 3:1. The solution was then incubated and gently stirred at 37 °C in an incubator overnight (20 h). When the reaction finished, the free cisplatin was removed by ultrafiltration using an Ultracel YM-3 system (Merck, Darmstadt, Germany) with a regenerated cellulose membrane, molar weight cutoff 3000. Aliquots of the albumin–cisplatin solution and the ultrafiltrate were analyzed for cisplatin content by UV absorption spectrometry as described previously (DeSimone et al. 1987). It was found that over 99% of cisplatin was bound to BSA.

Fluorescence measurement

The interaction of BSA–CP with PepGel was observed by fluorescence spectrophotometry. Fluorescence measurements were carried out on a fluorometer PAM 101-103 (H.Walz, Germany). Emission spectra were recorded from 300 to 450 nm with an excitation wavelength of 280 nm. For fluorescence measurements, peptide self-assembly was initiated in PBS solution and the final BSA–CP

concentration for this experiment was 1.5 wt% in 0, 0.1, 0.2 and 0.3 wt% PepGel.

Transmission electron microscopy (TEM) and confocal microscopy

The BSA–CP aggregation change following the addition of PepGel was imaged by a Philips CM 100 TEM (FEI Company, Hillsboro, OR). After a 1.5 wt% BSA–CP and 0.2 wt% PepGel mixture was kept in room temperature for 15 min, 10 µl of the mixture and 1.5 wt% BSA–CP solution samples were placed on a TEM grid for 1 min. The TEM grid sample was then negatively stained for 30 s with 2% (w/v) uranyl acetate. Excess staining solution was removed, and TEM was operated at an accelerating voltage of 100 kV.

Confocal fluorescence images of 1.5 wt% BSA–CP in PBS solution containing 0.1% BSA–FITC with/without 0.1 wt% PepGel mixture were taken on an LSM 5 PASCAL confocal microscope (Carl Zeiss Inc, Thornwood, NY, USA) at an excitation wavelength of 488 nm. Fluorescence was imaged with a 560LP filter.

Rheological tests

The storage (G') of hydrogels from PepGel was determined on a C-VOR 150 rheometer system (Malvern instruments, Malvern, Worcestershire WR141XZ, UK) with a 20-mm-diameter parallel plate geometry and a 500-mm gap size. Single-frequency (1 Hz) steady shear strain (1%) was used for testing. Samples with PepGel concentration 0.1, 0.2, or 0.3 wt% with 1.5 wt% BSA–CP were prepared as previously described regarding the PBS solution. 200 µl of gel-forming solution was placed on the measuring system immediately after BSA–CP solution was added. Subsequently, the elastic modulus (G') and viscous modulus (G'') as a function of time were monitored for 1 h at 37 °C.

BSA–CP release test

To determine BSA–CP release from PepGel, 0.1, 0.2, and 0.3 wt% PepGel mixing with 1.5 wt% BSA–CP were prepared in triplicate with PBS solution, and 500 µl of the hydrogel was pipetted into corning six-well transwell polyester membrane inserts (pore size 0.4 µm). The transwells were inserted into six-well plates and incubated at 37 °C for 15 min. Following this step, 3 ml of PBS was pipetted into wells, and the plates were kept in a humidified atmosphere at 37 °C. At each time point, 1 ml of the respective well PBS surrounding solutions was taken out and read on a UV-1650 pc spectrophotometer (Shimadzu, Kyoto, Japan). The absorbance peak at 280 nm was recorded for all the solutions measured and corrected by subtracting the background signal from PBS solution. The aliquots were returned to the wells after measurements. A release fraction was calculated by (I_t/I_∞), where I_t is the absorbance of the drug released at time, and I_∞ is the absorbance of drug released at infinite long time, which is absorbance of the total BSA–CP in the 500-µl PepGel hydrogel dissolved in 3.5 ml of PBS. Released fractions were averaged from three replicates for each sample, and mean values and standard errors were determined each time.

Cell culture and drug efficiency test

Hela cells were seeded in a 24-well plate (1.5×10^4 cells/well) and incubated in 700 µl of DMEM containing 10% FBS. 0.1, 0.2, and 0.3 wt% PepGel mixing with 1.5 wt% BSA–CP were prepared as described previously. 100 µl of these samples was pipetted into corning transwell polyester membrane inserts for 24-well plates (pore size 0.4 µm) and kept in an incubator in a humidified, 5% CO_2 atmosphere at 37 °C for 15 min. Then the inserts were immersed in the plate wells containing the seeded HeLa cells. The plates were incubated for 3 days in the incubator and the cell viability of every sample was made at least in triplicate.

A CCK-8 assay was used to determine cell viability after each treatment. 10 µl of CCK solution was added to each well. After 4 h of incubation in the incubator, 100 µl of the respective well cell culture solutions was taken out. Absorbance at 450 nm was collected in a 96-well microplate on a microplate reader (mQuant, Bio-Tek, USA) and corrected by subtracting the background signal from a wall containing only 100 DMEM with 10% FBS. The absorption intensities were averaged from three replicates for each sample and normalized by cells in the wells treated with 100 µl of PBS solution in the inserts to obtain the cell viability, and mean values and standard errors were determined each time.

Results and discussion

Fluorescence experiment

The interactions of cisplatin with BSA have been a frequent topic of study in structure–activity relationship research (Holding et al. 1991; Yotsuyanagi et al. 1991; Espósto and Naiiar 2002; Huang et al. 2014). The accurate mechanism of the interaction of cisplatin–albumin binding is still highly controversial due to the lack of profound understandings despite countless studies in the past few decades. The excessively large proportion of cisplatin binding to albumin could lead to albumin's structural and aggregation status change. It was found that when the amount of cisplatin bound per albumin exceeds five, the obvious content of

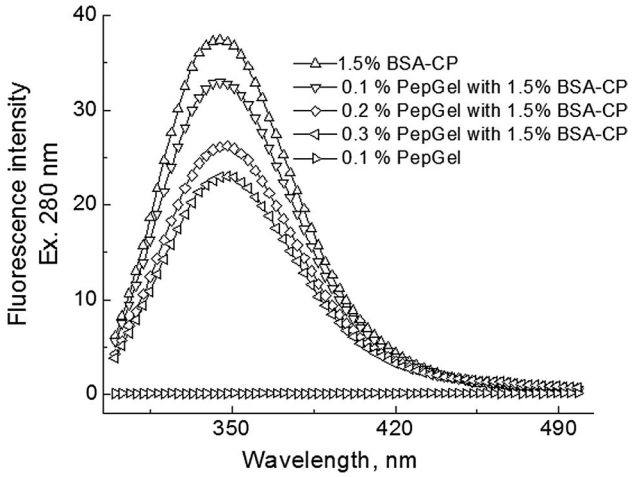

Fig. 1 Fluorescence spectra from 1.5 wt% BSA–CP encapsulated in 0, 0.1, 0.2, and 0.3 wt% hydrogel from PepGel

α-helix in albumin could be damaged (Huang et al. 2014). In addition, dimers and higher cross-linked forms of albumin would be formed, preventing the efficient delivery of metal drugs to tumor targets (Huang et al. 2014). In this study, we used a ratio of three cisplatin to one albumin to ensure the efficacy of cisplatin while maintaining, to the extent possible, the structure of BSA.

BSA is a widely studied protein and has been used as a model protein for diverse biophysical, biochemical, and physicochemical studies. The unique 3D structure of BSA was determined by X-ray crystallography. The structure is composed of three domains (I, II, and III), which confers a heart-shaped molecular structure and forms a large hydrophobic cavity that acts as a hydrophobic binding site (He and Carter 1992; Gelamo et al. 2002). The ability of BSA to bind with a number of hydrophobic ligands is well documented. Two tryptophan residues, namely Trp 135 and Trp 214, reside in BSA. Trp 214 is hidden in the hydrophobic loop; however, Trp 135 is more laid bare to a hydrophilic environment (Sulkowska 2002). The tryptophan in the hydrophobic cavity can act as an intrinsic fluorescence probe, and ligands to the hydrophobic cavity in BSA can quench the fluorescence from the tryptophan by energy transfer.

The fluorescent emission spectra of BSA–CP at excitation of 280 nm showed a maximum of 340 nm. Upon the addition of 0.1, 0.2, and 0.3 wt% of PepGel, the fluorescence intensity was suppressed by 14, 30, and 40%, respectively (Fig. 1). The fluorescence deduction is triggered by the energy transfer rather than the absorption by the peptide because the peptide absorption at 280 nm at the experimental concentration is negligible (data not shown).

Factors such as hydrophobic forces, electrostatic interactions, van der Waals interactions, and hydrogen bonds are at work in the interactions between a ligand and a protein. The hydrophobic sequence FLIVIGSII in the PepGel plays a key role in hydrogel formation by interacting with hydrophobic forces. Due to the apolar properties of amino acid I residues, it is expected that FLIVIGSII can penetrate into the protein matrix and preferentially attain the hydrophobic subdomain of albumin, causing a decrease in Trp fluorescence.

Distribution of BSA–CP in PepGel hydrogel

To study the role of PepGel in drug loading and release, BSA–CP was used as a model drug in this study as cisplatin is commonly used to treat cancers. Here, BSA–CP is not only a drug carrier but also a hydrogel trigger. There are two possible mechanisms through which BSA–CP could trigger the hydrogelation for PepGel: (1) by adjusting the electrostatic interactions among PepGel peptides, as some amino acids in BSA are ionic in aqueous solution, and (2) by acting as a linker and binding multiple sequences of FLIVIGSII through hydrophobic forces. In the previous experiment using fluorescence instruments, we have indicated that PepGel peptides can effectively bind to BSA–CP complexion. Thus, mechanism (2) is more theoretically possible.

The PepGel peptide can self-assemble into nanofibers with a lot of fragments. After being triggered, the fragments disappeared and longer fibers were observed, forming extensive cross-linking points and leading to the formation of hydrogel (images included in another work). To study the interaction between BSA–CP and PepGel, we imaged the morphological change of BSA–CP in PBS solution following the addition of PepGel solution. As is shown in Fig. 2a, in aqueous solution, BSA–CP tended to aggregate. After the addition of PepGel solution, the big clots disappeared and integrated into the network of PepGel fiber (Fig. 2b). This result indicated that BSA–CP could interact with PepGel fiber, in agreement with the data of fluorescence.

The distribution of BSA–CP was approximatively obtained using fluorescence probe BSA–FITC. After being dissolved in PBS solution, the BSA–FITC was found, through confocal fluorescence microscopy, to spread evenly (Fig. 2c). However, after the addition of PepGel, the network structure was found (Fig. 2d), indicating that the distribution of BSA–FITC was mainly on the PepGel fiber. Because of the minor difference between BSA–FITC and BSA–CP, it is reasonable to believe that BSA–CP has a similar distribution, i.e., on the PepGel fiber.

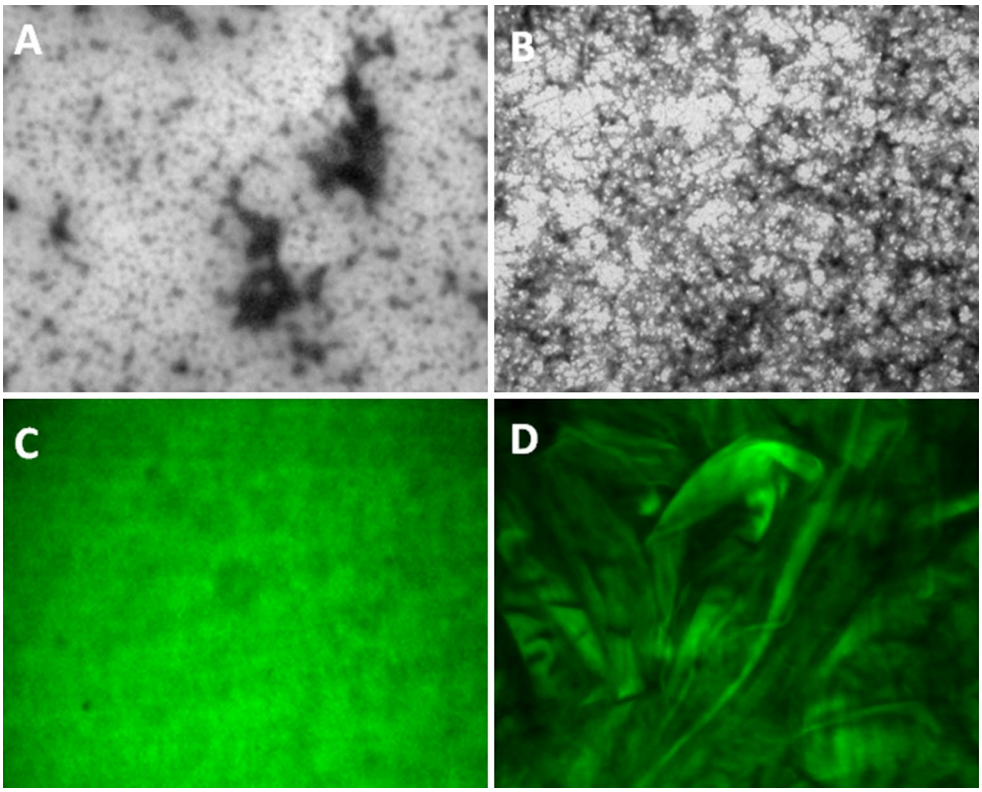

Fig. 2 TEM images of 1.5 wt% BSA–CP in PBS solution (**a**) and PBS solution containing 0.2 wt% PepGel (**b**); fluorescence confocal images of 1.5 wt% BSA–CP and 0.1 wt% BSA–FITC in PBS solution (**c**) and PBS solution containing 0.1 wt% PepGel (**d**)

Oscillatory rheology

The investigation of the mechanical properties of the hydrogel from PepGel triggered with BSA–CP revealed that rheometry determined the viscoelasticity and gelation behaviors. With regard to frequency, the elastic modulus (G') relies on the concentration of PepGel. With the increase of the concentration of PepGel, the storage modulus of hydrogel accelerated accordingly (Fig. 3a). The elastic moduli (G') at 1 Hz of hydrogel with the PepGel concentration 0.1, 0.2, and 0.3 wt% were 6, 39, and 87, respectively. As a result of concentration, the loss modulus (G'') increased as well, nevertheless the disparity was minor (Fig. 3b). The gel structure was characterized by means of the ratio of elastic modulus to loss modulus (G'/G''), and $G'/G'' = 1$ is deemed as a critical point of gelation. The ratios of elastic modulus to loss modulus (G'/G'') of hydrogel with all concentrations exceed 1 at 1 Hz. Even though the hydrogel with PepGel concentration 0.1 and 0. 2 wt% showed the relatively high values of G'/G'' (1.48 and 4.64, respectively), it did not show a self-supporting property. It was observed that G'/G''

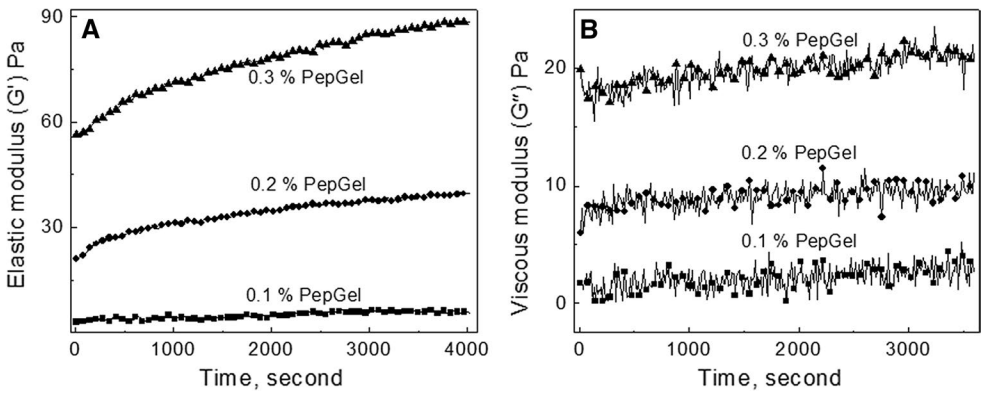

Fig. 3 Elastic modulus (G') (**a**) and viscous modulus (**b**) of 0.1, 0.2, and 0.3 wt% PepGel hydrogel in DMEM containing 1.5 wt% BSA–CP during hydrogelation at 37 °C

value of the hydrogel with PepGel concentration 0.3 wt% was 4.19 and that the formulation formed a self-supporting hydrogel.

BSA–CP load and delivery

In the fluorescence and rheology experiments, we have shown that PepGel could significantly suppress the fluorescence for Trp in the BSA–CP and trigger the formation of hydrogel, indicating that BSA–CP could interact with PepGel peptide and be loaded into hydrogel. In the TEM experiment, we have imaged that BSA–CP could form aggregates in PBS buffer solution, which may be the reason why BSA–CP was released in a slow manner. As shown in Fig. 4, for BSA–CP in PBS solution, around 60% of the drug complexion was released on the first day; in contrast, the release rates of BSA–CP for the 0.1, 0.2, and 0.3 wt% PepGel were 49, 47, and 41%, respectively. Release profiles throughout the course of the measurement period indicated that the hydrogel from PepGel could effectively reduce the release of BSA–CP.

BSA triggers the gelation of PepGel by acting as a linker, which binds multiple sequences of FLIVIGSII in the PepGel peptides and enhances the hydrophobic interaction. Massive releases of BSA–CP will inevitably lead to the collapse of hydrogel and the decrease of the concentration of PepGel in the inner insert. However, after 6 days by the end of the measurement period, we found that the 0.3 wt% PepGel was still stable gel, which meant that the decrease of peptide was not at a significant level. Therefore, the interaction of BSA–CP with the hydrophobic functionalities on PepGel peptide is believed to be strong and even 40% of the remaining BSA–CP could keep the status of hydrogel.

HeLa cell viability

The therapeutical effect of albumin-bound platinum on cancer is controversial. An early study by Takahashi et al. has shown that cisplatin–albumin only demonstrates antitumor activity at very high concentrations of cisplatin–albumin and drew the conclusion that it is unlikely for the complex to contribute to the antitumor activity of cisplatin in vitro (Takahashi et al. 1985). However, numerous studied have shown that BSA–CP did have some beneficial activities; in addition, it alleviated side effects such as nephrotoxicity or ototoxicity (Holding et al. 1991, 1992). In this study, using the complex with a ratio of 3 cisplatin to 1 albumin, it has been found that cisplatin–BSA possessed obvious effects against HeLa cancer cells.

Figure 5 shows the in vitro cytotoxic effect of BSA–CP from 0, 0.1, 0.2, 0.3 wt% PepGel for HeLe cells. The drug loading for BSA–CP was 1.5 wt%. We can see from this figure that the HeLe cells achieved higher viability following the higher concentration of PepGel and the cell viability for samples with varying concentration of PepGel followed a sequence as 0.3 wt% > 0.2 wt% > 0.1 wt % > 0. We have indicated that pure PepGel did not influence the propagation of HeLa cells (included in another work). Thus, the increase of HeLa cell viability in the function of PepGel indicated the inhibition of BSA–CP release by hydrogel from PepGel.

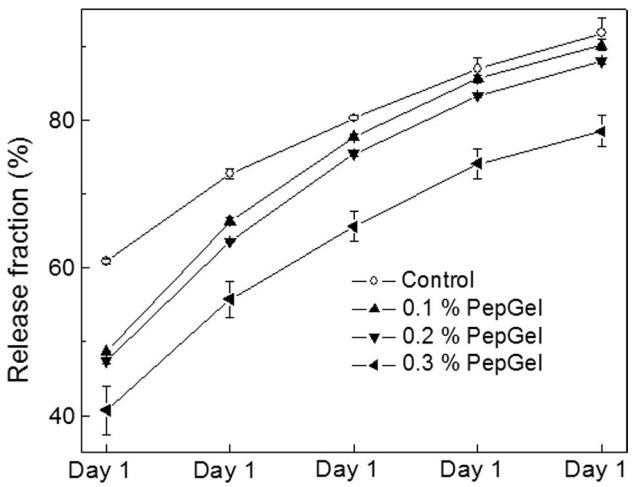

Fig. 4 Fraction release of BSA–CP as a function of time from 0, 0.1, 0.2, and 0.3 wt% hydrogel from PepGel at 37 °C

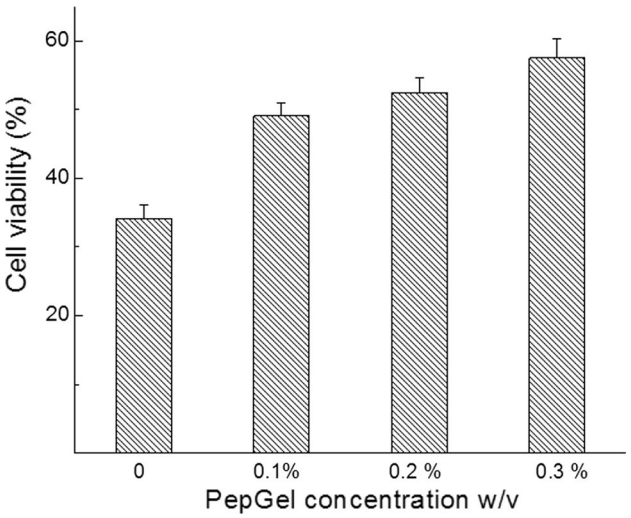

Fig. 5 Cell viability of HeLa cells treated with 1.5 wt% from 0, 0.1, 0.2, and 0.3 wt% hydrogels from PepGel for 3 days

Conclusion

In conclusion, BSA–CP/PepGel network was fabricated via in situ hydrogelation induced by hydrophobic force. Physical association and distribution of BSA–CP in the nanofibers were observed by TEM and fluorescence confocal images. Furthermore, hydrogel from PepGel can load and inhibit the diffusion of BSA–CP and thus act as a carrier of controlled releases of BSA–CP complex even at very low concentrations (below 0.3 wt%). The performance of PepGel was confirmed by in vitro experiments using HeLa cells. This research provided evidence that PepGel can be an effective carrier for BSA-bound or -linked drugs.

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Author contribution statement JL designed and conducted the experiments, GL and JW analyzed the results, XSS designed experiments and data interpretation. All authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Research involving human participants and/or animals This article does not involve any studies with human participants or animals performed by any of the authors.

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