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Research Article

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Preparation of a drug carrier through supramolecular hydrogel based on poly(ethylene glycol) methyl ether- folic acid - α-cyclodextrin for controlled delivery of Doxorubicin

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Abstract This study is aimed at developing novel, inexpensive hydrogels for containing drug and with the potential for targeting cancer cells *in vitro.* In this work, Folic acid has been conjugated with α-Cyclodextrin and then used as a building block for constructing novel supramolecular hydrogels together with Poly(ethylene glycol) methyl ether in aqueous solutions. Resultant hydrogel materials have been investigated with respect to their controlled release characteristics for the encapsulated drug Doxorubicin hydrochloride and biological activity was also studied for this supramolecular hydrogels.

Keywords Drug delivery, Doxorubicin, Folic acid, supramolecular hydrogel, α-Cyclodextrin, Poly(ethylene glycol) methyl ether

Introduction

Supramolecular physical hydrogel formation can be induced in aqueous media. The process is driven by molecular self-assembly, therefore does not require additional cross-linking reagents, and the transition from solution to gel occurs without a significant change in volume [1-3]. The inclusion complex formation capability of CDs has only recently been utilized as a non-covalent binding motif for the development of a wide variety of dynamic polymeric networks and assemblies in aqueous media [4-6]. These polymeric systems have been frequently investigated for pharmaceutical and biomedical applications including the sustained and targeted release of bioactive substances, biocompatible scaffolds for tissue engineering and medical diagnostics. In recent years, research in supramolecular CD polymeric hydrogels has been broadly developed [7]. Although supramolecular hydrogels with cyclodextrins as hosts possess interesting properties for the delivery of therapeutics with high pharmacological activity, low therapeutic index, and poor physicochemical properties, they cannot be used for active drug targeting because they are devoid of any specificity for biological structures. To exploit cyclodextrins as targetable drug delivery systems, the oligosaccharide structure should be properly functionalized with targeting moieties such as peptides, hormones, vitamins, antibody fragments, etc.

Folic acid is a small vitamin, which interacts specifically with the folate binding protein (FBP) located in the caveole-like invaginations on the cell surface receptor [8-9]. Upon receptor interaction, the folate acid-FBP complex is taken up by cells and moves through the many organelles involved in endocytotic trafficking, providing for cytosolic deposition [8]. The folic acid receptor is overexpressed by many types of tumor cells, including ovarian, endometrial, colorectal, breast, lung, renal, neuroendocrine carcinomas, and brain metastases [10]. The folate receptor mediated endocytosis has been largely investigated to expand the therapeutic value of drugs, by increasing delivery to the target tissue as well as the target/nontarget tissue ratio. When folic acid (FA) is attached to carboxyl site, the folate retains its normal receptor-binding affinity and therefore, can be internalized by receptor mediated endocytosis [11]. This principle has been exploited for the selective delivery of imaging agents [12], gene [13]

therapeutic agents [14], micelles of block copolymers [15], and other complexes of macromolecular [16] to tumor/cancer cells.

Doxorubicin hydrochloride is a drug widely used in cancer chemotherapy [17-18]. However, Dox is a strong cytotoxic compound for normal tissues and produces extensive biochemical adverse effects on the physiology of the patient. To decrease the toxicity of Dox, targeted delivery of the drug through supramolecular hydrogels would be an alternative efficient option for cancer therapy.

In my previous work, folic acid has been conjugated with the α-CD and then used for constructing supramolecular hydrogels together with Poly(ethylene glycol) methyl ether in aqueous solutions [19]. This strategy can result in the formation of bioactive supramolecular hydrogels with multifunctional characteristics. In this work, resultant hydrogel materials have been investigated with respect to their controlled release characteristics for the encapsulated drug Doxorubicin hydrochloride and biological activity was also studied for this supramolecular hydrogels.

Materials and Methods

Materials

Folic acid (FA), N-hydroxysuccinimide (NHS), 1-ethyl-3-3(3-dimethylaminopropyl) carbodiimide (EDC), Poly(ethylene glycol) methyl ether (MPEG) with molecular weight of 5000, Bovine serum albumin (BSA), and Doxorubicin hydrochloride (Dox) were purchased from Sigma-Aldrich. α-Cyclodextrin was purchased from Acros. Cell culture medium Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced Salt Solution, trypsin-EDTA (0.25%), penicillin G, streptomycin sulfate, fetal bovine serum (FBS) and trypan blue stain were purchased from Invitrogen Corporation (Grand Island, NY, USA). All other solvents and analytical reagents were purchased from commercial suppliers and used as received.

Methods

Supramolecular hydrogels were prepared by the inclusion complexation of MPEG with α-CD-FA in an aqueous solution. In a typical experiment, the required amount of MPEG (1, 2 or 3 wt %) was dissolved in an aqueous solution and then mixed with an aqueous α-CD-FA solution (6, 7 or 8 wt %) at room temperature [19].

In vitro cytotoxicity of Dox-loaded supramolecular hydrogelation

Cell culture maintenance: Human hepatocellular carcinoma (Hep-G_2) cells were maintained in Eagle's Minimum Essential Medium (EMEM) with Earle's Balanced salt solution, supplemented with 1.5 g/l sodium bicarbonate, 2 mM l-glutamine, 0.1mM non-essential amino acids, 1mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) fetal bovine serum (FBS) and grown at 37^{o}C in a humidified atmosphere and in the presence of 5% CO_2. The culture medium was changed and the cells were tripsinized when confluence reached approximately 80-90%.

In vitro cytotoxicity of Dox-loaded supramolecular hydrogelation: The quantification of the cytotoxic effect of the free Dox, hydrogel system of MPEG with α-CD-FA, Dox-loaded hydrogelagainst Hep-G_2 cells was investigated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In brief, the cells were seeded on 96 microplates at concentration of 5×10^4 cells/well in 100 µl of EMEM media and were grown in a humidified incubator at 37^{o}C with 5% CO_2 for 24h. Then the culture medium was replaced by EMEM without increasing the FBS containing concentrations, then incubated at 37^{o}C. After 24h, when the cells had adhered, hydrogel solutions with or without Dox and free Dox at different concentrations (0, 0.3, 3, 30, 300 µg/ml) were incubated separately with the cells for 24h. Following incubation, cells were washed twice with the culture medium and 100µl of EMEM medium added with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (10 µl) 5.0 mg/ml in sterile-filtered PBS to each well, and the plates were re-incubated for another 2.5 h. The yellow MTT was reduced to purple formazan in the mitochondria of living cells. The formazan crystals were dissolved in DMSO and then the MTT solution was removed by aspiration, after which MTT crystals were solubilized with 100µl dimethyl sulfoxide (DMSO). The optical density (OD) of each sample was assessed at 540 nm using a dual wavelength reader (Dynatech, Denkendort, Germany). Data are present as multiples of control. The untreated cells were used as the

control. The values are the mean of 8 replications for each treatment. The absorbance of each well was normalized with their respective controls. The cell viability was calculated as follows:

$$\text{Cell viability (\%)} = \frac{B}{A} \times 100$$

where A is the absorbance of the cells incubated with the culture medium (control) and B is the absorbance of the cells incubated with the drug-loaded hydrogels or free drug (treatments).

Preparation of Dox-encapsulated in supramolecular hydrogel and *in vitro* release

For the in situ encapsulation of Dox, 0.2% Dox was first dissolved in an aqueous α-CD-FA solution, and the required amount of MPEG aqueous solution was then added to induce supramolecular gelation at room temperature (25^{o}C). To study the in vitro release behavior, a total of 1.0ml of solution formulation was injected into a 10 ml tube and then set overnight for hydrogel formation. Phosphate buffer saline (PBS, 4.0 ml, 0.01 mol/l, pH 7.4) was added to the tube as the release medium. The tube was incubated in a shaking incubator with a 60 stroke/min at 37^{o}C during the test. At a given time point, 2.0 ml of supernatant was collected from each tube, which was then replaced by the same amount of fresh prewarmed PBS. The time for each sampling was determined to be 30 s, in which the mixture did not approach the equilibrium. UV-Vis spectroscopy was used to record the Dox content using a JASCO V-670 spectrophotometer at 482nm. The percentage of cumulative amounts of Dox released was calculated using the standard calibration curve. All release studies were carried out in triplicate.

Results and Discussion

***In vitro* toxicity study**

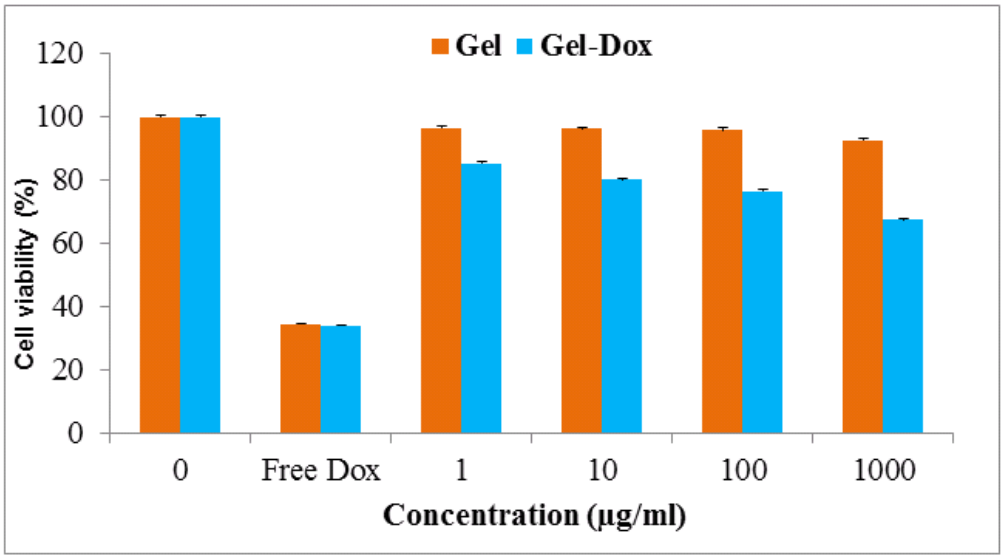

Figure 1: In vitro cell viability of free hyrogel and hydrogel encapsulated Dox formulations against Hep-G_2 cells after 24 h of incubation

Estimation of the potential toxicity for polymer based materials is extremely important for drug delivery applications. For this reason we determined toxicity from the MTT method. The viability of Hep-G_2 cells after 24h of incubation in a culture medium with the supramolecular gelation and Dox loaded supramolecular gelation was determined by using MTT method, as shown in Figure 1. Overall, free Dox was shown to be dose-dependent with the most pronounced cytotoxic effect at 30 and 300µg/ml more effective than other doses taken in our study after 24 hrs of incubation time. We fixed 300µg/ml as the amount for use in later experiments due to the potential effect of this dose on this cell line. From the results shown in Figure 1, it can be seen that the Dox loaded in the supramolecular gelation showed higher cytotoxicity than the free hydrogel unloaded Dox at 24h. More interestingly,

the free supramolecular gels obtained from MPEG and α-CD-FA showed almost no signs of cytotoxicity even in higher concentrations. The results indicate that this supramolecular gel was nontoxic. It is believed that the excellent biocompatibility can be attributed to the following possible reason: that the Dox may affect the α-CD molecules threading onto the MPEG chains during the gelation formation. These concentrations were well above the amounts typically used for drug delivery systems.

***In vitro* release behavior of encapsulated Dox from MPEG/α-CD-FA supramolecular hydrogels**

The potential of MPEG/α-CD-FA supramolecular hydrogel for use in an injectable drug delivery system for the encapsulation and sustained release of a bioactive compound was investigated. For this purpose, Dox was used as a model drug and was encapsulated in the hydrogel matrix during the preparation process. In each case, the original concentration of encapsulated Dox was the same. Figure 2 shows the in vitro release profiles for encapsulated Dox released from MPEG/α-CD-FA supramolecular hydrogels with different compositions in a pH 7.4 PBS at 37°C. Depending on the amount of MPEG or α-CD-FA used for the hydrogel formation, various release rates were found for the encapsulated Dox. A higher amount of MPEG or α-CD-FA could prolong the Dox release profile of the supramolecular hydrogel. In other words, the Dox release rate decreased with the increase of MPEG or α-CD-FA amount. In contrast, the amount of α-CD-FA affected the drug release behavior more strongly than did the amount of MPEG. This is because α-CD-FA plays a key role in the host-guest interactions, enabling the formation of supramolecular hydrogel networks.

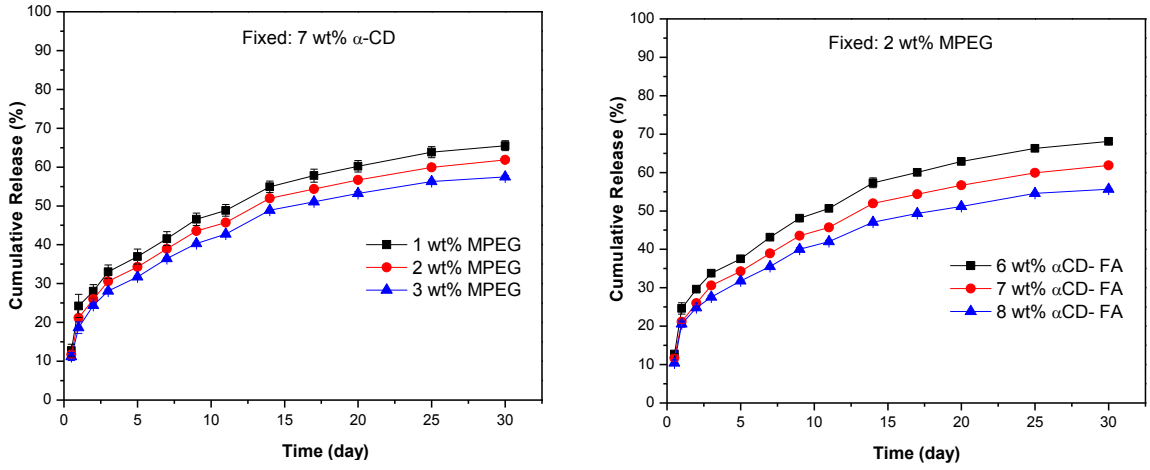

Figure 2: In vitro cumulative release of encapsulated Dox from MPEG/α-CD-FA supramolecular hydrogels with different compositions: (a) effect of MPEG amount; (b) effect of α-CD-FA amount

To understand the release mechanism of encapsulated Dox, we fitted the accumulative Dox release data using the following semiempirical equation [20]:

$$M_t/M_\infty = Kt^n \qquad (\text{for } M_t/M_\infty \leq 0.6),$$

where M_t and M_∞ are the cumulative amount of the drug released at t and equilibrium, respectively; K is the rate constant relating to the properties of the hydrogel matrix and the drug; and n is the release exponent characterizing the transport mechanism. According to this classification, there are four distinguishable modes of diffusion:

(i) the value of n = 0.5 suggests Fickian or Case I transport behavior in which the relaxation coefficient is negligible during transient sorption;

(ii) the value of n = 1 indicates to a non-Fickian or Case II mode of transport where the morphological changes are abrupt;

(iii) if 0.5 <n <1, the transport process is anomalous, corresponding to Case III, and the structural relaxation is comparable to diffusion;

(iv) a value of n <0.5 indicates a pseudo-Fickian behavior of diffusion where the sorption curves resemble Fickian curves, but the approach to final equilibrium is very slow.

By plotting $\log(M_t/M_\infty)$ versus log(t), the n and K values as well as the corresponding determination coefficients (R^2) were obtained, as listed in Table 1. The K values were found to decrease with the increase of MPEG or α-CD-FA amount. This phenomenon could be attributed to the formation of a denser hydrogel network in the case of a higher MPEG or α-CD-FA amount, which hindered the release of high molar mass Dox from the supramolecular hydrogel. In addition, the n values in all cases were found to be smaller than 0.5, showing the pseudo-Fickian behavior of the diffusion release mechanism and the approach to final equilibrium was very slow.

Table 1 Release charac:teristics of encapsulated Dox from MPEG/α-CD-FA supramolecular hydrogels with different compositions*

Hydrogel compositions	K	n	R^2	Transport mechanism
Effect of MPEG amount				
1 wt% MPEG + 7 wt% α-CD-FA	1.313	0.362	0.964	Case IV
2 wt% MPEG + 7 wt% α-CD-FA	1.271	0.375	0.971	Case IV
3 wt% MPEG + 7 wt% α-CD-FA	1.237	0.378	0.979	Case IV
Effect of α-CD-FA amount				
6 wt% α-CD-FA + 2 wt% MPE	1.320	0.37	0.961	Case IV
7 wt% α-CD-FA + 2 wt% MPE	1.271	0.375	0.971	Case IV
8 wt% α-CD-FA + 2 wt% MPE	1.239	0.368	0.956	Case IV

** Other conditions: 0.2 wt% Dox, pH 7.4, 37* °C.

Conclusion

The hydrogel system reported in this work is a new approach for the development of supramolecular hydrogels. The encapsulated Dox shows a controlled and sustained release behavior that would be advantageous for use as an injectable hydrogel matrix for drug delivery and cell encapsulation. This study provides a novel drug-entrapment strategy for hydrophilic hydrogel-based carriers.

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