Original Article

Thai Journal of Pharmaceutical Sciences

Synthesis and evaluation of poly(ethylene glycol) diacrylate-modified chitosan as a mucoadhesive polymer

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AQ1 **Received:** ???
Accepted: ???
Published: ???

ABSTRACT

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This study aimed to synthesize a poly(ethylene glycol) diacrylate-modified chitosan (CS-PEGDA) as a mucoadhesive polymer for transmucosal drug delivery. Chitosan-cysteine (Cys-CS) was synthesized by coupling chemistry. Cys-CS was then employed for the synthesis of CS-PEGDA through a thiol-ene click reaction between the thiol groups of Cys-CS and alkene groups of PEGDA using triethanolamine as an activator. The obtained structure of the synthesized polymer was proved using Fourier transform infrared (FT-IR) spectroscopy and nuclear magnetic resonance (NMR) spectroscopy. The mucoadhesive capability of the synthesized polymer was examined by an *ex vivo* mucoadhesion test using a texture analyzer. The toxicity of synthesized polymer was also investigated by an methylthiazolyldiphenyl-tetrazolium (MTT) assay on normal human gingival fibroblast (HGF) cells. The NMR and FT-IR spectra of the synthesized polymer ensured the expected structure of the polymer and the presence of acrylate moieties on the backbone of the CS-PEGDA, which play a crucial role in mucoadhesion process. The obtained CS-PEGDA exhibited superior mucoadhesive properties compared with the intact CS. These results could demonstrate the potentials of CS-PEGDA as a mucoadhesive material for transmucosal drug delivery systems.

Keywords: Chitosan, cysteine, mucoadhesion, poly(ethylene glycol) diacrylate, thiol-ene reaction

INTRODUCTION

Mucoadhesive drug delivery systems can maintain contact of the delivery systems with mucous membrane.[1] These delivery systems could provide a prolonged retention time and a release of a drug at the targeted site, leading to an enhancement in the bioavailability of the drug locally and systemically.[2] Mucoadhesive polymers are the polymers that can form strong interaction with mucin glycoprotein.[3] These polymers can be categorized into three subgroups: Polymers are viscid when contacted with water, polymers that form weak or strong interactions with mucin, and polymers that particularly bind to a receptor site.[4] Thiol-containing polymers (thiomers) are second-generation mucoadhesive materials that were extensively reported since 1990s.[5] Thiomers are able to generate covalent bonds with cysteine-rich subunits of mucous membrane by disulfide bond formation.[6] The bonds formed between thiomers and mucin are stronger than ionic interactions, hydrogen bonds, or van der Waal's forces generated by conventional polymer with the mucous membrane.[7] Unfotunately, thiomers are comparatively less stable due thiol oxidation at pH >5 which lead to the decrease in the interactions with mucus membrane resulting in reduced efficacy. On the other hand, synthetic polymers containing acyclic moieties had also been reported as mucoadhesive materials.[8] Acrylate-conjugated polymer could adhere to mucosal surfaces through Michael-type reaction which provides strong mucoadhesion force.[9] In particular, acrylated polymers were shown to be more stable than thiolated polymers.[10] Most studied materials used in mucoadhesive drug delivery systems are the polymers derived from chitosan (CS). CS is a widely used polymer with a carbohydrate backbone comprising of N-acetyl-d-glucosamine and d-glucosamine repeating units linked by (1-4)-β-glycosidic bond.[11] Thiolated CS polymers are generated by conjugation of the CS amine with thiol-containing molecule such ascystein, 2-iminothiolane, or thioglycolic acid.[12-14] Delivery systems prepared from thiolated CS that had been reported to be able to form disulfide linkages with cysteine residues on mucin resulting in

an increased adhesion.[15,16] Thiol-ene click reactions, which are rapid reactions generated under mild aqueous conditions, refer to the reaction of alkenes with thiol-containing compounds.[17] The high efficiency of thiol-ene reactions has been clarified in the synthesis and functinalization of various macromolecular and polymeric materials.[18] In this study, poly(ethylene glycol) diacrylate-modified CS(CS-PEGDA) was synthesized through a thiol-ene click reaction to improve the mucoadhesive competence of CS. The successful synthesis was verified by proton nuclear magnetic resonance spectroscopy (1H NMR) and Fourier transform infrared (FT-IR) spectroscopy. Moreover, the mucoadhesive capability and the cytotoxicity of the polymer were also appraised.

MATERIALS AND METHODS

Materials

CS (molecular weights 8000 g/mol) was procured from OliZac Technologies Co., Ltd. (Bangkok, Thailand). Triethanolamine (TEA) was received from P.C. Drug center Co., Ltd. (Bangkok, Thailand). Dulbeccos modified Eagles medium (DMEM) was purchased from Gibco BRL (Rockville, MD, USA). Poly(ethylene glycol) diacrylate (PEGDA, M_n 250), L-cysteine hydrochloride, N-hydroxysuccinimide, 5,5-Dithiobis (2-nitrobenzoic acid) (Elman's Reagent), MTT bromide, porcine buccal mucosa (Type II), and N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride were attained from Sigma-Chemical Co. (St. Louis, MO, USA). The reagents used in the assay were used without further purification and were of analytical reagent grade.

Synthesis of Cysteine-grafted CS Chitosan-cysteine (Cys-CS)

Cys-CS was synthesized following the previously reported procedure.[19] The synthesized polymer was then examined by ^{1}H NMR (AVANCE III HD, Bruker). The synthetic pathway of Cys-CS is shown in Figure 1. Ellman's assay was employed for the quantitation of the thiol moieties on the Cys-CS.[12] Briefly, accurately weighed Cys-CS samples were dispersed in microcentrifuge tubes containing 500 µL of 0.5 M phosphate buffer (pH 8). After that, 500 µL Ellman's reagent (5,5'-dithiobis [2-nitrobenzoic acid] or DTNB) solution (0.3 mg/mL) was included in the dispersed sample. The mixture was mixed and kept in the dark for 90 min prior to the absorbance detection at 450 nm. The amount of thiol groups was computed from a standard curve obtained by Ellman's assay of a series of standard cysteine hydrochloride solution with increasing concentration of cysteine hydrochloride.

Synthesis of Poly(ethylene glycol) Diacrylate-conjugated CS (CS-PEGDA)

Cys-CS was employed in the synthesis of CS-PEGDA. Briefly, Cys-CS was dissolved in ethanol contained in a round-bottom flask, and the pH was adjusted to 3.0–4.0. PEGDA (0.25–1.25 mmol/g of Cys-CS) was then added to the polymer solution. Afterward, TEA (0.5 µmol) was filled in the polymer mixture. The polymer mixture was fused for 18 h by a magnetic stirrer at room temperature to allow thiol-ene formation. Thereafter, the unreacted compounds were eliminated by dialysis against DI water for 7 cycles (the water was changed every 4 h) before lyophilization. The synthetic pathway of CS-PEGDA is displayed in Figure 2.

FT-IR Spectroscopy

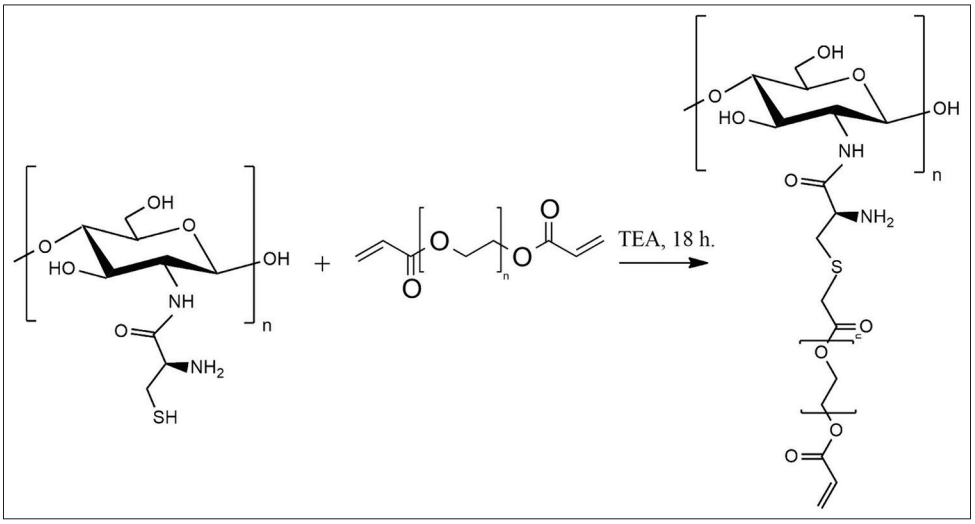
The chemical structures of the synthesized polymer were confirmed by FT-IR using a Nicolet 4700 infrared spectrophotometer (wavenumber range 400–4000 cm^{-1}) at a resolution of 4 cm^{-1} and 16 running scan.

^{1}H NMR Spectroscopy

The complete functionalization of the polymer was verified at 298 K by NMR spectrometer 300 MHz (AVANCE III HD, Bruker). δ_{D2O} = 4.80 ppm as the solvent of CS and Cys-CS and δ_{DMSO} = 2.50 ppm as solvent of PEGDA and the synthesized polymers.

Ex vivo Mucoadhesive Study

To measure the mucoadhesion strength of the synthesized polymer, *ex vivo* mucoadhesion experiment of the compressed



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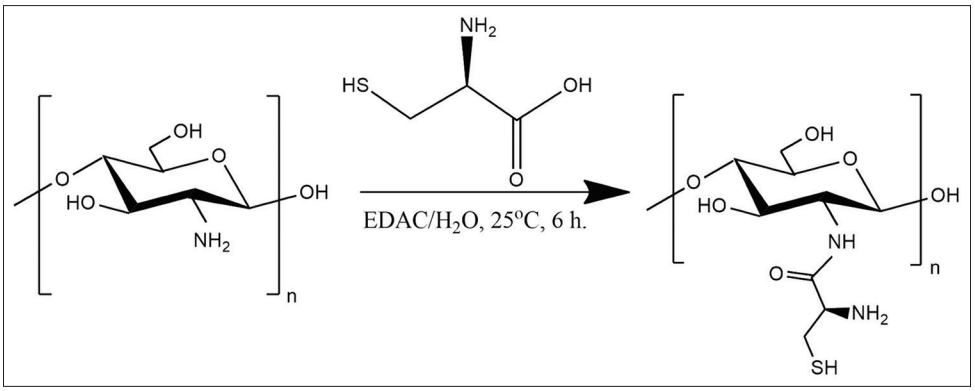


AQ3 **Figure 2:** ???

polymers tablets was determined on porcine buccal mucosa.[19] In brief, the synthesized polymer (50 mg) was compressed into a small, round tablet. The porcine buccal mucosa was employed as the mucous tissue. The tablet was attached with a cylindrical Perspex probe of a texture analyzer (5 kg-load cells) (TA.XTPlus, Stable Micro Systems, Hamilton, USA). The excised tissue was adhered to the testing platform. Next, 500 µL of artificial saliva (2.38 g Na_2HPO_4, 8 g of NaCl and 0.19 g KH_2PO_4 in 1000 ml of deionized water; pH 6.8) was spread on the mucosa surface. The speed of probe was 2 mm/s for 15 sec with a force of 0.3 N. The maximum force used to pull the compressed tablet off the mucosa was determined as mucoadhesion strength.

Cytotoxicity

The cell viability of HGF cells after being contacted with the synthesized polymer and CS were verified using MTT assay. HGF cells in DMEM were allocated into 96-well plates (10,000 cells/well). The cells were kept at 37°C with 95% air and 5% CO_2. Then, the cells in DMEM without serum were cultivated with the synthesized polymers at the concentrations between 0.1 to 1,000 µg/mL for 24 h. Afterward, the cells were rinsed with PBS pH 7.4 and fill 100 µL DMEM medium with serum and MTT solution 25 µL (0.5 mg/mL). After 3 h, the precipitate of formazan formed after incubation was dissolved by adding DMSO (100 µL) and the absorbance was detected at 550 nm (VICTOR Nivo™, Perkin Elmer, MA, USA). The calculation of % relative cell viability was displayed in Equation 1.

$$\text{Relative cell viability}\,(\%) = \frac{\text{Abs}_{550,\ \text{sample}} - \text{Abs}_{550,\ \text{blank}}}{\text{Abs}_{550,\ \text{control}} - \text{Abs}_{550,\ \text{blank}}} \times 100 \quad (1)$$

Statistical Analysis

Three replications of all experiments were conducted. Data are shown as mean ± standard deviation. The statistical analysis used independent T-test and F-tests at 95% confidence interval using Microsoft® Excel 2019 ($P < 0.05$).

RESULTS AND DISCUSSION

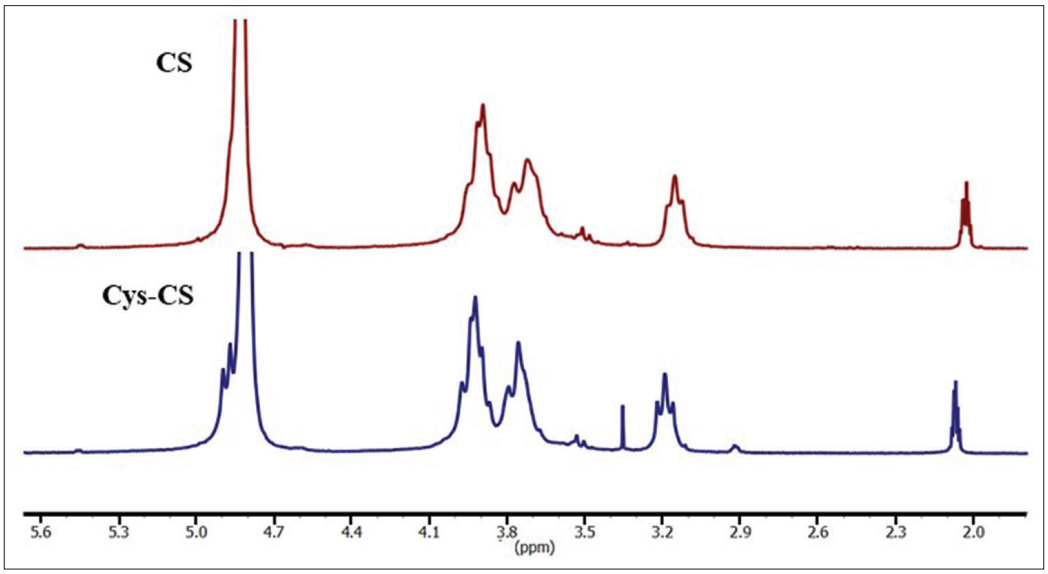
Synthesis of Cysteine-grafted CS (Cys-CS)

The ^{1}H NMR spectrum of CS in Figure 3 displayed a signal from 3.0 to 3.3 ppm which was represented by C2 glucosamine ring's H-bond. The signals at 3.4 and 4.1 ppm are attributed to the H-bond of C3, C4, C5, and C6 on the glucopyranose ring. The addition signal at 3.35 ppm was observed in the spectrum of Cys-CS. This finding is relevant to the results reported in the previous study.[4] The thiol content of the synthesized Cys-CS was 87 µmol/g.

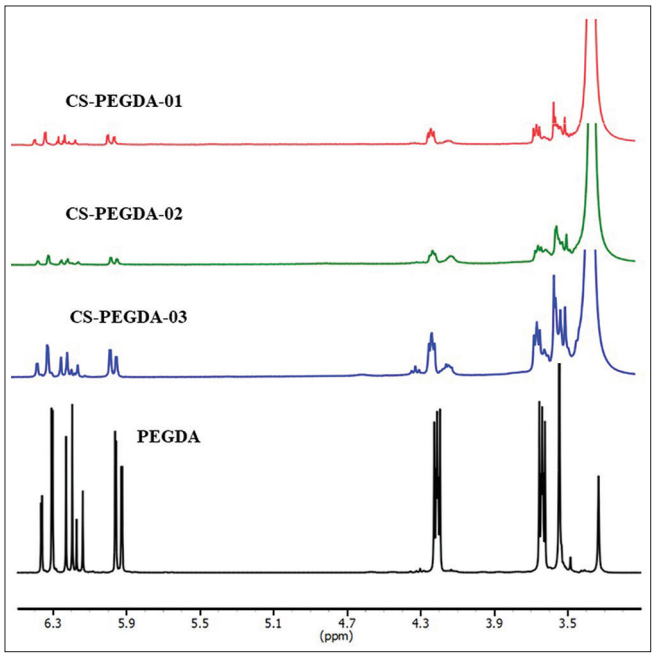
Synthesis of Poly(ethylene glycol) Diacrylate-modified CS (CS-PEGDA)

The CS-PEGDA was synthesized by thiol-ene reaction between acrylate functional groups of PEGDA and thiol groups of Cys-Cs. The amount of PEGDA used in the synthesis reaction was varied (0.25–1.25 mmol/g of Cys-CS) which could affect the synthesis yield and the quantity of acrylate presented on the CS backbone. Further, increase in the PEGDA amount to be more than 1.25 mmol resulted in the precipitation of Cys-CS. Therefore, the concentration of PEGDA used in the synthesis was in the range of 0.25–1.25 mmol/g of Cys-CS. The % yields of the CS-PEGDA polymer obtained after the synthesis reaction using different amounts of PEGDA are listed in Table 1. Increasing the amount of PEGDA in the reaction led to the increase in the %yield of CS-PEGDA which the CS-PEGDA-03 provided the highest yield. Therefore, this formulation was then selected for further experiments.

The accomplished conjugation of PEGDA to the Cys-CS was verified by FT-IR and ^{1}H NMR. Figure 4 displays the ^{1}H NMR spectra of CS-PEGDA and PEGDA. The ^{1}H NMR spectra of the PEGDA shows signals at 3.64 and 4.21 ppm, which represents the protons of ethylene glycol. The signals at 5.94, 6.19 and 6.33 ppm correspond to the proton of diacrylate structure. After poly(ethylene glycol) diacrylate was conjugated to the Cys-CS structure, the proton peaks of methylene end groups of ethylene glycol were found at δ = 3.6–4.0 ppm and the proton peaks the acrylate vinyl end group were found at δ = 5.9–6.3. The FT-IR spectra of CS, Cys-CS, CS-PEGDA-01, CS-PEGDA-02, CS-PEGDA-03, and PEGDA are illustrated in Figure 5. The broad peak at around 3300 cm^{-1} due to the O-H bond was found in the spectrum of Cys-CS. The spectrum of PEGDA presented the sharp peak at around 1700 cm^{-1} which indicates the presence of carbonyl groups. The peak at 1640 cm^{-1} was attributed to -C=C- bonds of acrylate. The spectrum of the synthesized



AQ3 **Figure 3:** ???



AQ3 **Figure 4:** ???

CS-PEGDA also showed that the peaks found in Cys-CS and PEGDA. In addition, the peak intensity of -C=C- bonds increased as the amount of PEGDA increased. These could be an indication of successful conjugation of PEGDA onto the CS backbone.

Ex vivo Mucoadhesive Study

The pulling force used to detach the polymer tablets from *ex vivo* porcine buccal mucosa is expressed as *ex vivo* mucoadhesion strength. The mucoadhesion strength of CS-PEGDA-03 is assessed compared with CS and Cys-CS, and the results are displayed in Table 2. CS-PEGDA-03 exhibited almost 3-fold improvement in the mucoadhesive capability compared

Table 1: Synthesis yields of CS-PEGDA

Formulation	Amount of PEGDA per gram of Cys-CS	% yields
CS-PEGDA-01	0.25 mmol	25.0
CS-PEGDA-02	0.75 mmol	35.2
CS-PEGDA-03	1.25 mmol	48.7

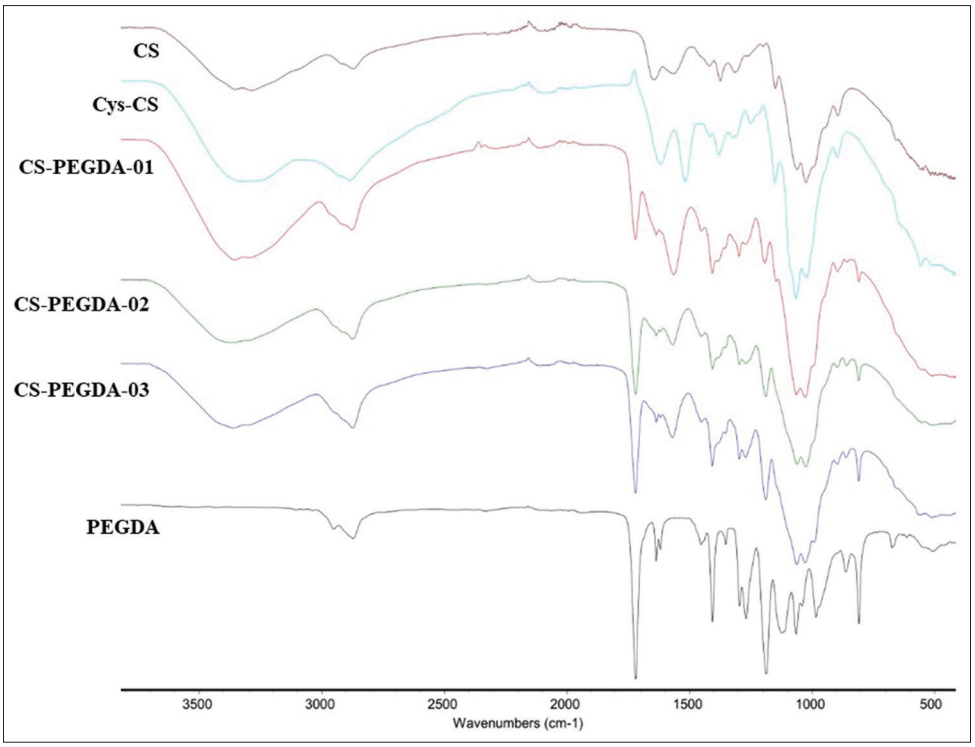
Table 2: *Ex vivo* mucoadhesive strength of CS-PEGDA-03 compared to Cys-CS and Chitosan

Polymer	Ex-vivo mucoadhesive strength (*n*)
Chitosan	0.027±0.006
Cys-CS	0.074±0.017
CS-PEGDA-03	0.073±0.007

to the intact CS. These results imply that CS-PEGDA can produce more entanglements due to the bulky PEGDA chain, together with the formation of hydrogen and covalent bonds with mucous membrane.[20] In addition, the mucoadhesive strength was comparable to the Cys-CS which is a well-known mucoadhesive polymer. Although the CS-PEGDA provided comparable mucoadhesive properties to Cys-CS, acrylate functionality was found to be more stable. Disulfide bond formation between the thiol group of a thiolated polymer due to oxidation could lead to the reduced mucoadhesiveness of the polymer.[21]

Cytotoxicity

The cytotoxicity of CS-PEGDA-03 and CS was estimated using an MTT test. Figure 6. represents the % cell viability of HGF cells treated with CS-PEGDA-03 compared with CS. As it can be seen from the graph, CS-PEGDA-03 was nontoxic to the HGF cells with the range of concentration up to 1000 µg/mL. Further, *in vivo* toxicity testing in animals should be performed to obtain toxicity data for the clinical application of this polymer.



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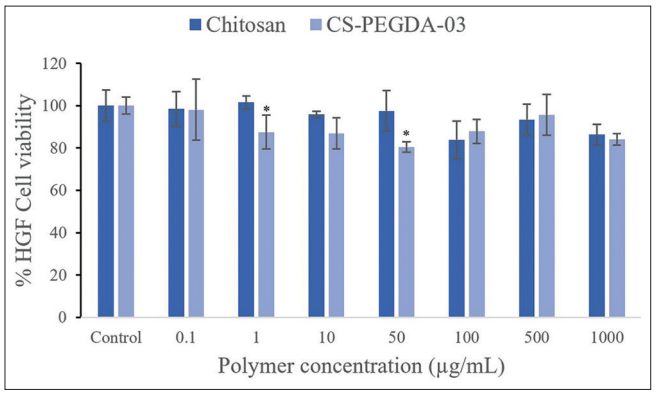

AQ3 **Figure 6:** ???

CONCLUSION

A new mucoadhesive polymer, CS-PEGDA, has been successfully produced by a thiol-ene click reaction between the Cys-CS and poly(ethylene glycol) diacrylate. The synthesized polymer exhibited excellent mucoadhesive properties which were comparable to Cys-CS and much greater than the intact CS. Moreover, the CS-PEGDA had low toxicity to HGF cells at the polymer concentration of 0.1–1000 µg/mL. Consequently, the CS-PEGDA is probably a material that can be used as a drug-delivering material for mucoadhesive drug delivery systems.

ACKNOWLEDGMENT

The Thailand Research Funds (RTA6180003) and the Research and Creative Fund, Faculty of Pharmacy, Silpakorn University are acknowledged for the funding supports.

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