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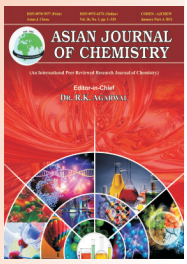

in vitro and *in vivo* Evaluation of Novel Ocular Delivery System of 5-Fluorouracil Peptide Hydrogel

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A novel biocompatible hydrogel was prepared based on the supramolecular self-assembly of a peptide containing a bioactive RGD (arginine-glycine-aspartic acid) sequence and a hydrophobic N-fluorenyl-9-methoxycarbonyl (FMOC) tail. The *in vitro* biocompatibility assay demonstrated that such self-assembled peptide hydrogel had a well biocompatibility. When intraoperatively administrating the self-assembled peptide hydrogel containing 5-fluorouracil in the filtering surgery of rabbit eyes, quantitative determination of 5-fluorouracil in the aqueous humor showed detectable amounts of 5-fluorouracil (below 1μg/mL) for 1 weeks, which shows that peptide hydrogel was still present in the subconjunctival space for that period and released 5-fluorouracil in a continuous fashion. Attributed to the sustained release of 5-fluorouracil from the hydrogel, the injection of the self-assembled peptide hydrogel containing 5-fluorouracil at surgery site had potential advantages including preventing the frequent 5-fluorouracil injections and the toxicity of 5-fluorouracil to the surrounding ocular tissues efficiently, indicating a potential application of the self-assembled peptide hydrogel as an implanted drug delivery system for the treatment of scarring formation after filtering surgery.

Keywords: Self-assembly, Peptide hydrogel, Filtering Surgery, Ocular Delivery System.

INTRODUCTION

Filtering surgery is a conventional treatment for glaucoma after medical and laser therapies have failed[1]. Such strategy usually involves generating a filtration fistula to allow escape of aqueous humor from the anterior chamber into the subconjunctival space to lower intraocular pressure (IOP). Generally, the success rate of glaucoma-filtering surgery is limited by the formation of postoperative scarring[2]. 5-fluorouracil (5-FU) and mitomycin C (MMC) are widely used in clinical practice to prevent postoperative scarring and improve the success rate of glaucoma-filtering surgery[3,4]. However, late bleb leaks, bleb ruptures and infections may frequently occur after using 5-fluorouracil and mitomycin C[5,6]. Especially, 5-fluorouracil have been reported with higher incidences of punctate corneal epitheliopathy and conjunctival epithelial defects owing to multiple subconjunctival injections after filtering surgery[7,8].

The side effects of injected 5-fluorouracil after the glaucoma-filtering surgery can be reduced by using subconjunctivally implanted drug delivery systems that could provide a localized and sustained release of antiproliferative drugs over an extended period.

Hydrogels are formed with the networks of hydrophilic polymers which can swell in water and hold a large amount of water while maintaining the chemical structures[9-12]. Due to the similarity between the highly hydrated three-dimensional networks and the hydrated body tissues, hydrogels have been widely used in biomedical fields, including drug delivery systems[13] and tissue engineering scaffolds[14].

In recent years, researches on hydrogels have expanded rapidly, such as the build block comprising of a specific peptide sequence and a hydrophobic aromatic tail, which has an ability to self-assemble into supramolecular hydrogel by taking advantage of π-π stacking interactions[15,16]. The self-assembled peptides and their derivatives are the most attractive hydrogelators due to their well-biocompatible and biodegradable properties.

In this study, a 5-fluorouracil (FU) ocular delivery system was prepared using supramolecular hydrogel based on the self-assembled process of peptide containing a novel bioactive RGD (arginine-glycine-aspartic acid) sequence and a hydrophobic FMOC (N-fluorenyl-9-methoxycarbonyl) tail. Based on our study, the *in vitro* biocompatibility assay demonstrated that such self-assembled peptide hydrogel had a well biocompatibility.

The preparation can control drug release *in vitro* for a certain period. Meanwhile, *in vivo* drug release characteristics were determined to evaluate the potential of the prepared 5-fluorouracil peptide hydrogel as a complement system in glaucoma filtration surgery.

EXPERIMENTAL

5-Fluorouracil peptide hydrogel preparation: Peptide (FMOC-FFRGDF) was synthesized *via* the FMOC-based peptide solid synthesis technique. N-Fluorenyl-9-methoxycarbonyl (FMOC) protected L-amino acids (FMOC-Gly-OH, FMOC-Asp(OtBu)-OH, FMOC-Arg(Pbf)-OH, FMOC-Phe-OH) and 2-chlorotrityl chloride resin (100-200 mesh, loading: 1.32 mmol/g) were purchased from GL Biochem (Shanghai) Ltd. (China) and used as received. Piperdine, trifluoroacetic acid (TFA), N-hydroxy-benzotriazole (HOBt), dimethylsulfoxide (DMSO) and *o*-benzotriazole-N,N,N',N'-tetramethyluronium-hexafluorophosphate (HBTU) were provided by Shanghai Reagent Chemical Co. (China) and used directly. Dimethyl formamide (DMF) and diisopropylethylamine (DiEA) were obtained from Shanghai Reagent Chemical Co. (China) and distilled prior to use. Triisopropylsilane (TIS) was purchased from ACROS (USA) and used without further purification. All other reagents and solvents were of analytical grade and used directly. For the 5-fluorouracil peptide hydrogel preparation, the mixture of FMOC-FFRGDF and 5-fluorouracil powder were well dissolved in ultra purified water to form 15 mg/mL peptide solution containing 5 wt. % 5-fluorouracil (5 % based on the amount of peptide used) and subsequently filtrated for the sterilization. After placing at room temperature for 0.5 h, a well defined 5-fluorouracil peptide hydrogel appeared based on the self-assembly of the peptide molecules.

***in vitro* Drug release:** The self-assembled peptide hydrogel containing 5-fluorouracil (5 wt % based on the amount of peptide used) was first prepared in cylindrical glass vial with only the top surface exposed for release. Thereafter, 1 mL distilled water was added to the top of the self-assembled peptide hydrogel. At a predetermined time interval, the entire volume of water above the self-assembled peptide hydrogel was removed and 1 mL fresh distilled water was added after each sampling. Each time interval was performed in triplicate and the experiments were carried out at room temperature for 1 week. The amount of 5-fluorouracil released from the self-assembled peptide hydrogel was measured by using a UV spectrophotometer (Perkin-Elmer Lambda Bio 40 UV/visible spectrometer, USA) at 265 nm. The cumulative drug release was calculated as: Cumulative amount released (%) = $(M_t/M_\infty) \times 100$, where M_t is the amount of 5-fluorouracil released from the self-assembled peptide hydrogel at time t and M_∞ is the amount of 5-fluorouracil loaded in the self-assembled peptide hydrogel.

Cell culture: HeLa cells were incubated, respectively in DMEM containing 10 % FBS and 1 % antibiotics (penicillin-streptomycin, 10,000 U/mL) at 37 °C in a humidified atmosphere containing 5 % CO_2. Then, cells were harvested and diluted in cell media to 6×10^4 cells/mL. Before seeding cells, the aqueous solution of peptide was prepared as described above and transferred to 24-well plastic culture plate to form a self-assembled peptide hydrogel. Then 500 µL cells in DMEM with 10 % FBS (3×10^4) were suspended on the surface of the self-assembled peptide hydrogel. After incubation for 72 h, the cell adhesion and proliferation were observed by using an inverted-light microscope (Olympus IX 70). The microscopy images were obtained at the magnification of 100 × and recorded using CoolSNAP-Pro (4.5.1.1) software. The control experiment was carried out in 24-well plastic culture plate without the self-assembled peptide hydrogel.

***in vitro* Cytotoxicity assay:** The *in vitro* cytotoxicity of peptide was examined by MTT assay. HeLa cells were seeded, respectively in the 96-well plate with a density of 6000 cells/well and incubated for 24 h in 200 µL Dulbecco's Modified Eagle Medium (DMEM) containing 10 % fetal calf serum (FBS). After the addition of peptide for 72 h, the medium was replaced with 200 µL fresh DMEM. Then 30 µL MTT solution (5 mg/mL) was added. After incubation for 4 h, 200 µL DMSO was added and the mixture was shaken at room temperature. The optical density (OD) was measured at 570 nm with a microplate reader, model 550 (BIO-RAD, USA). The cell viable rate was calculated as: Viable rate (%) = $(OD_{treated}/OD_{control}) \times 100$, where $OD_{control}$ is obtained in the absence of peptide and $OD_{treated}$ is obtained in the presence of peptide.

Animals: Japanese albino rabbits weighing between 2.5 and 3.5 kg were used in this study (laboratory animal center, Tongji Medical College, Huazhong University of Science and Technology, China). The experiments were conducted in accordance with the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Eight eyes were used in experimental group.

Animal study: General anesthesia was induced with intramuscular injection of 50 mg/kg ketamine and 15 mg/kg xylazine. With rabbits under local anesthesia with dicaine 1 % (Union Hospital, Wuhan, China).

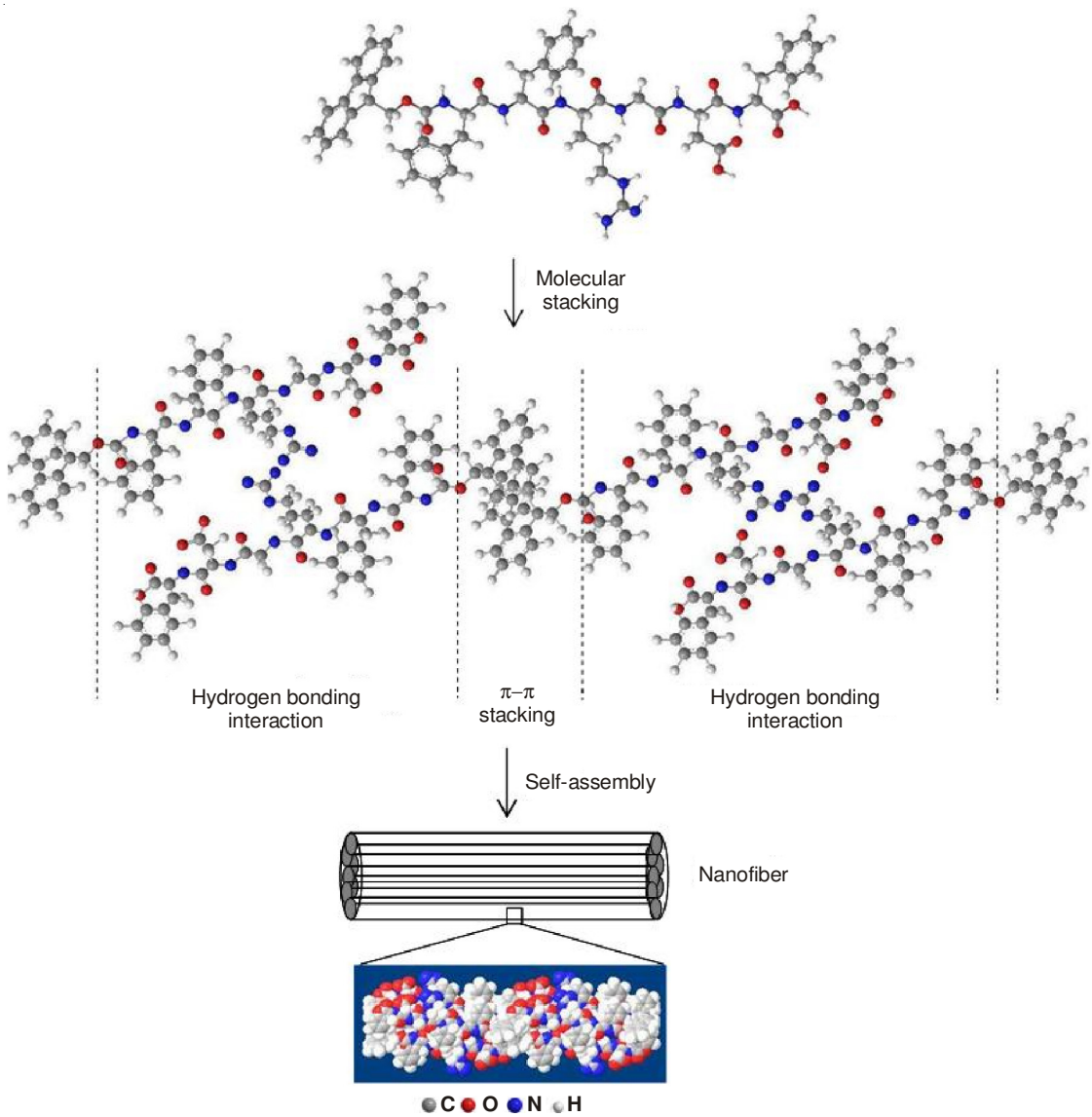
The surgical procedure was performed under a dissecting microscope with coaxial-light illumintion and a variable magnification of 2 ± 10 × (Topcon, Japan). A lid speculum was inserted to expose the globe and a limbus-based conjunctival flap was reflected. Tenonectomy was performed to expose the underlying sclera, followed by careful conjunctival dissection anterior to the limbus. Hemostasis was carefully maintained with cautery. A half-thickness, limbal-based, 4 × 4 mm scleral flap was made that extended just anterior to the limbus. A 3 mm limbal incision was made with a 45° blade that entered the anterior chamber. A block of tissue containing inner sclera, trabeculum and peripheral cornea, measuring approximately 3 × 1 mm, was excised at the limbus. A peripheral iridectomy was then performed. The scleral flap was approximated with two 10-0 nylon sutures (Alcon Laboratories, Inc, USA). The conjunctiva was repositioned and the wound closed with 8-0 Vicryl suture (Ethicon, Inc, USA) in a continuous fashion. Just before the last step, a 0.9 mm needle (20 gauge) was inserted in the subconjunctival space and 200 µl of 5-fluorouracil peptide hydrogel was injected adjacent to the site of filtering surgery. The suture was closed tightly and topical cyclomycin ophthalmic ointment was applied. Filtering surgery and injection of 5-fluorouracil peptide hydrogel were performed on both eyes of the rabbit.

Preparation of tissue samples: Concentrations of 5-fluorouracil were determined by liquid chromatography (LC) using a high-performance liquid chromatography (HPLC) system (LC module I plus; Waters, Milford, MA), consisting of a power line controller pump (model 600E) an autoinjector (715 Ultra WISPTM), a tunable absorbance UV detector set at 268

nm and a software integrator (Millennium) all from Waters. Separation was performed with a C18 HPLC column (Nucleosil 100-5; Macherey Nagel, Düren, Germany; 250 × 4 mm; internal diameter, 5 µm). The mobile phase consisted of ammonium phosphate buffer (50 mM; pH 6.0) delivered at 1 mL/min. Before use, the mobile phase was filtered through a 0.45 mm membrane filter (Millipore, Molsheim, France) and degassed 10 min by sonication. At postoperative days 1, 2, 5, 7 and 14, a paracentesis was made with rabbits under local anesthesia and 100 mL of aqueous humor was collected for quantitative analysis. Samples and quantitative standards were homogenized and directly injected into the HPLC system, with an injection volume of 20 mL.

RESULTS AND DISCUSSION

Synthesis of peptide: Solid phase synthesis (SPPS) which was first developed by Merrifield *et al.*[17] is a process by which chemical transformations can be carried out on solid support to prepare a wide range of synthetic compounds. In comparison with the solution phase peptide synthesis, this strategy provides several advantages such as efficiency, convenient work-up and purification procedures. In this study, solution phase peptide synthesis technique was employed to prepare the peptide by using four kinds of amino acids including phenylalanine (Phe), glycine (Gly), aspartic acid (Asp) and arginine (Arg). Through the repetition of acylation reaction with a sequence of Phe, Asp, Gly, Arg, Phe and Phe, the peptide was obtained and the corresponding molecular structure is presented in Fig. 1a. There was a bioactive RGD sequence, the well-known cell adhesion ligand found in fibronectin. Besides, a hydrophobic FMOC tail was also incorporated into the peptide, which can provide the π-π stacking interactions during the supramolecular self-assembly of the peptide. The HPLC profile of the prepared peptide in Fig. 1b indicated a high purity of the peptide (99.1 %). And ESI-MS data in Fig. 1c demonstrated the validity of the peptide structure. **Scheme-1**. Illustrated the supramolecular self-assembly of the peptide resulting from the superhelical arrangement of the peptide sequences *via* hydrogen bonding inte-raction and the π-π stacking of fluorenyl groups in FMOC tails.



Scheme-I: Schematic illustration of the supramolecular self-assembly of the peptide resulting from the superhelical arrangement of the peptide sequences *via* hydrogen bonding interaction and the π-π stacking of fluorenyl groups in FMOC tails

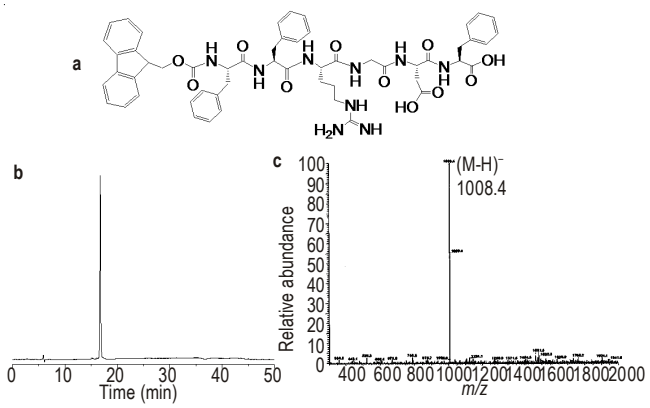


Fig. 1. (a): Molecular structure of the peptide; (b) and (c): HPLC and ESI-MS profiles of the peptide, respectively

***in vitro* Drug release:** One of the great benefits to use a self-assembling system for therapeutic delivery is that the therapeutic can be incorporated during the formation of material. This strategy allows precise control over the concentration of the incorporated drug. In this study, after the formation of the self-assembled peptide hydrogel containing 5-fluorouracil, the corresponding release profile was investigated and presented in Fig. 2. About 21.7 % incorporated 5-fluorouracil was released at the 1st day. And the cumulative drug release increased to 44.2 % at the 3rd day. After the sustained release for 7 days, the incorporated 5-fluorouracil could be completely released from the self-assembled peptide hydrogel. From the data in Fig. 2, it was found that 5-fluorouracil was gradually released from the self-assembled peptide hydrogel and no burst release was observed. This unique property made the self-assembled peptide prepared in this study having a potential to use as a valuable drug delivery system for the application in biomedical field.

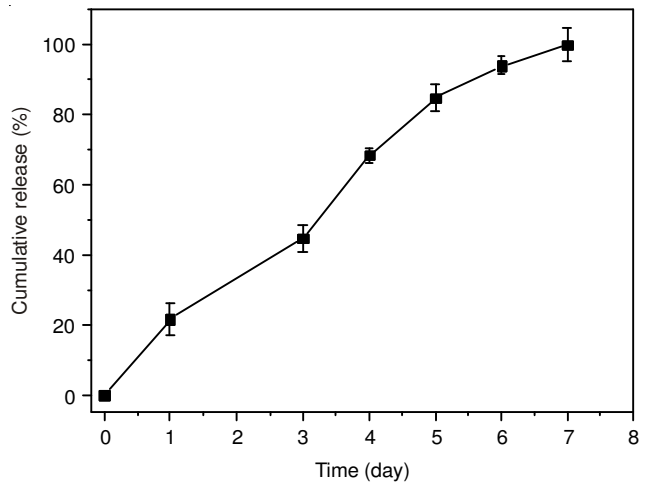


Fig. 2. Cumulative release of 5-FU at room temperature from the self-assembled peptide hydrogel

***in vitro* Biocompatibility assay:** It is known that peptide based scaffolds containing bioactive RGD segments represent an important kind of extracellular matrix (ECM) that can support cell adhesion and proliferation[18-20]. In order to investigate whether the newly self-assembled peptide hydrogel can serve as scaffold for tissue engineering application, the corresponding *in vitro* biocompatibility was assessed. Fig. 3a displays the viability of HeLa cells in the presence of the peptide. It was found that the cell viability rate kept around 100 % within the concentration range from 0 to 2.5 mg/mL, indicating that the peptide had no apparent cytotoxicity. To further investigate the ability of cells to adhere to the self-assembled peptide hydrogel, HeLa cells were suspended on the surface of the self-assembled peptide hydrogel after its formation in a 24 well culture plate. After incubation for 72 h, the adhesion and proliferation of cells were observed and presented in Fig. 3c. In comparison with the control experiment (Fig. 3b), all the cells can adhere to the self-assembled peptide hydrogel surface and proliferate. In addition, the elongated shape of the adhered cells was nearly identical to the cells that were incubated in non-gel coated wells, which demonstrated that the self-assembled peptide hydrogel had a well biocompatibility and can be applied as scaffold for tissue engineering application.

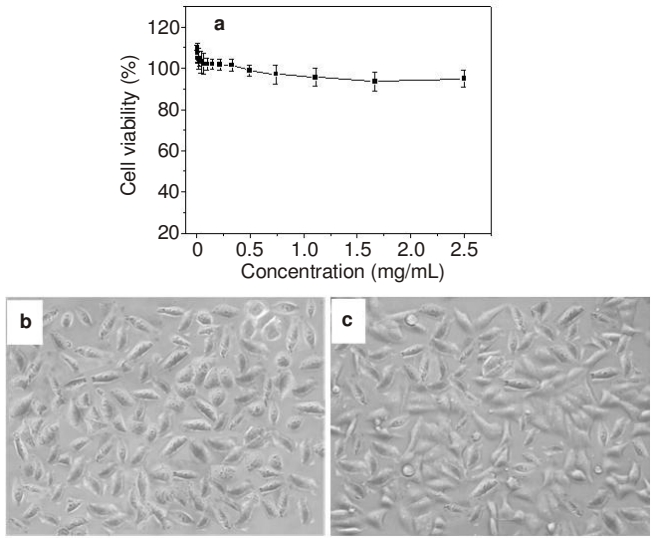


Fig. 3. (a): Cytotoxicity study of the peptide with different concentration; (b) and (c): HeLa cells cultured on a plastic cell culture plate and the surface of the self-assembled peptide hydrogel for 72 h, respectively

HPLC analyses: The quantitative determination of 5-fluorouracil concentration in the aqueous humor is particularly important to ensure that the levels reached are not toxic to the corneal endothelium. Corneal toxicity includes epithelial defects, corneal opacification and vascularization. The limit of quantitation of the established HPLC method was about 0.25 µg/mL for determining 5-fluorouracil in aqueous humor. There was no significant interfering substance after sample treatment.

From HPLC chromatograms of 5-fluorouracil, we can see time peak of 5-fluorouracil internal standard was 4.327 min, meaning 0.25 µg/mL for determining 5-fluorouracil concentration in aqueous humor (Fig. 4a). In eyes that received 5-fluorouracil peptide hydrogel, quantitative determination of 5-fluorouracil in the aqueous humor showed detectable amounts of 5-fluorouracil (below 1 µg/mL) for 1 weeks, which shows that peptide hydrogel was still present in the subconjunctival space for that period and released 5-fluorouracil in a continuous fashion. In particular, no burst release of 5-fluorouracil was observed in the early postoperative period. The time courses

of 5-fluorouracil concentration in aqueous humor, after implanting 5 wt. % 5-fluorouracil peptide hydrogel in the conjunctival region, are shown in Fig. 4. The aqueous humor levels have a maximum concentration at the initial time point (1 day). Other time point had points had 5-fluorouracil levels between 0.2 to 1 mg/mL. Thus, at each time point, a minimal amount of 5-fluorouracil was present in the anterior chamber, corresponding at least to 3 orders of magnitude below the threshold concentration for 5-fluorouracil toxicity to the corneal endothelium (1-10 mg/mL) reported by Mannis *et al.* [21].

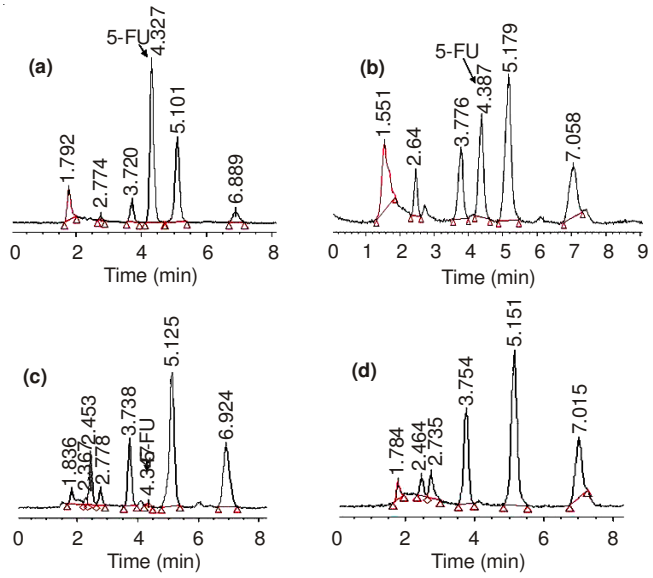


Fig. 4. HPLC Chromatograms of 5-Fluorouracil (5-FU). (a): 5-FU internal standard (0.25 µg/mL), (b): HPLC Chromatograms of 5-FU after filtering surgery at 1st day. (c): HPLC Chromatograms of 5-FU after filtering surgery at 7th day. (d): HPLC Chromatograms of 5-FU after filtering surgery at 14th day

Conclusion

In present study, this novel kind of drug delivery system based on RGD motif peptide hydrogel may have potential for glaucoma filtering surgery. Quantitative determination of 5-fluorouracil in the rabbit aqueous humor showed detectable amounts of 5-fluorouracil (below 1µg/mL) for 1 weeks, which shows that peptide hydrogel was still present in the subconjunctival space for that period and released 5-fluorouracil in a continuous fashion, offering potential benefits to avoid the frequent subconjunctival injections and decrease the toxic side effects in patients who are at high risk of failed filtering surgery.

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