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Original Article

FITC labeling of human insulin and transport of FITC-insulin conjugates through MDCK cell monolayer

Darshana Shah [a], Yuxing Guo [b], Joseph Ocando [b], Jun Shao [b, *]

[a] Avion Pharmaceuticals, 1880 McFarland Parkway, Suite 110-B, Alpharetta, GA, 30005, USA
[b] Department of Pharmaceutical Sciences, College of Pharmacy and Health Sciences, St. John's University, 8000 Utopia Parkway, Queens, NY, 11439, USA

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ABSTRACT

Fluorescein isothiocyanate-labeled insulin (FITC-insulin) has been widely used for bioanalytical applications. Due to the high cost of commercial FITC-insulin and tedious labeling procedures described in the literature, there is still a need to develop a cost effective, reliable and quick labeling method for insulin. The purpose of the present work was to develop a quick and affordable method for FITC labeling of human insulin and to determine the effect of different conjugations of FITC to human insulin on its permeability through the MDCK cell monolayer. FITC labeling of insulin gives mono-, di- or tri-conjugates depending on the reaction time and the molar ratio of FITC:insulin. Mono-conjugate with unlabeled insulin, mixture of di- and tri-conjugate, and tri-conjugate with very little amount of di-conjugate were synthesized in less than 4 h. Degree of conjugation had an effect on the permeability of insulin through the MDCK cell monolayer. Mono-conjugate had higher permeability than the unlabeled insulin due to increase in partition coefficient. However, tri-conjugate showed lower permeability than the unlabeled insulin due to the increase in molecular weight.

1. Introduction

Fluorescent probes are less expensive, much safer, more stable and much easier to dispose than radioisotopes, and hence they have gained much attention over the last several decades [1]. Fluorescein isothiocyanate (FITC), one of such probes, is widely used for labeling proteins and peptides due to its high detection sensitivity and low molecular size which has potentially low impact on the protein/peptide's biological activity [2].

FITC is an amine reactive fluorescent probe which labels biomolecules by forming a covalent bond between its isothiocyanate group and the primary and secondary amine groups of biomolecules [3]. FITC (MW 389.4) labeling of human insulin (MW 5807.57) may give mono-, di- and tri-conjugates of insulin since insulin has three amine reactive sites [4]. Insulin has been previously labeled with FITC and characterized for a number of applications such as protein tracing, protein-protein interaction, analytical detection, microsequencing, drug release study, permeability and cellular uptake study by fluorescence microscopy [4–8]. However, these reported labeling methods are tedious, which takes more than 12 h to produce different conjugates of FITC-insulin. Labeling method developed by Jacob et al. and Hentz et al. [4,6] takes 20 h or more to develop mixture of di- and tri-conjugate of FITC-insulin. The reported protocols for labeling are not robust. FITC-insulin is also commercially available and has been used by researchers, but is comparatively expensive [8,9]. Hence, there is still a need to develop an easy, quick and reliable labeling method to prepare different conjugates of FITC-insulin. Therefore, one of the aims of the present work was to study the reaction conditions to produce different conjugates of FITC-insulin and to develop a relatively simple and robust method.

The conjugation at different sites affects the biological activity of insulin. The biological activity of the FITC-insulin conjugates has been reported to differ from that of the native insulin. Hentz et al. [6] studied the biological activity of each conjugate and found that mono-conjugated FITC-insulin (tagged at B1 position) had the same biological activity as the native insulin whereas A1 conjugated FITC-insulin showed 10% decrease in the biological activity and di- and tri- conjugated FITC-insulin showed 100% decrease in biological activity as compared to the native insulin. The different conjugates may also affect the permeability of insulin due to the difference in molecular weight, hydrophilicity and lipophilicity. To

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* Corresponding author.
E-mail address: shaoj@stjohns.edu (J. Shao).

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our knowledge, no reports to date have been published regarding the effect of different conjugation on the permeability of FITC-insulin across a biological membrane. Therefore, the second aim of the present study was to investigate the impact of different FITC conjugates on the hydrophilicity and lipophilicity of insulin and its permeability through a cell monolayer.

2. Materials and methods

2.1. Materials

Human insulin and FITC-insulin (Human) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Fluorescein isothiocyanate isomer I, 95% (FITC) was purchased from Alfa Aesar (Tewksbury, MA, USA). PD-10 Sephadex™ G-25 M columns were purchased from GE Healthcare (Buckinghamshire, UK). Dulbecco's modified Eagle's medium (DMEM), Hyclon Hank's 1X balanced salt solution (HBSS), penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Fisher Scientific (Middletown, VA, USA). Transwell® inserts were purchased from VWR International (Allison Park, PA, USA). Human insulin ELISA kit was purchased from Crystal Chem (Elk Grove Village, IL, USA). MDCK cell line was purchased from ATCC® (Rockville, MD, USA).

2.2. Methods

2.2.1. FITC labeling of human insulin

FITC solution in DMSO (5 mg/mL) was added dropwise with gentle stirring to human insulin solution (15 mg/mL) in 0.1 M bicarbonate buffer (pH 9.5) with the molar ratio of FITC:insulin varying from 0.25:1 to 5:1. The reaction mixture was protected from light and kept at room temperature for 0.5–4 h with continuous slow stirring. Then the reaction mixture was incubated at room temperature for 30 min without stirring. The reaction mixture was then passed through a PD-10 Sephadex™ G25 column to separate the unbound FITC from insulin and FITC-insulin conjugates. Phosphate buffer (pH 7.4) was used to elute the mixture from the column and the eluent was collected in 32 fractions (fraction volume – 0.5 mL). Each fraction was analyzed by HPLC to quantify and determine the labeling efficiency. The fractions containing the conjugates were combined together and lyophilized overnight. The lyophilized FITC-insulin powder was stored at -20 °C for further use.

2.2.2. RP-HPLC assay

The reversed-phase HPLC (RP-HPLC) assay of insulin and FITC-insulin was carried out on an Agilent 1260 system equipped with an Agilent 1260 series UV detector and an Agilent 1220 series fluorescence detector. The analysis conditions were as follows: Column: C_{18} (4.6 mm × 150 mm, 5 μm); flow rate: 1 mL/min; injection volume: 20 μL; UV detector wavelength: 220 nm; fluorescence detector wavelengths: excitation – 495 nm, emission – 525 nm; mobile phase A – deionized water with 0.1% trifluoracetic acid (TFA); mobile phase B – 90% acetonitrile and 10% deionized water with 0.1% TFA; run time: 45 min. Gradient elution was used as follows for analysis: 0–15 min (15%–35% B), 15–25 min (35%–65% B), 25–32 min (65% B), 32–33 min (65%–100% B), 33–43 min (100% B) and 43–44 min (100%–15% B).

2.2.3. Lipophilicity and hydrophilicity assay of the conjugates

The lipophilicity and hydrophilicity of the conjugates were determined by the analysis of the partition coefficient (P). The method described by Griesser et al. [10] was adopted with slight modification. In brief, buffer (pH 7.4) containing 1 mg/mL of either FITC-insulin conjugates or unlabeled insulin was added to 1-octanol (1:1) and incubated for 8 h at 37 °C while shaken at 300 rpm. The samples were centrifuged by centrifuge for 10 min at 9,000 rcf. Aliquots (100 μL) were withdrawn from both the phases and analyzed for absorbance and fluorescence by HPLC. Partition coefficient (P) was calculated as the ratio of the insulin concentration in the octanol phase vs in the aqueous phase.

2.2.4. Transport of FITC-insulin through MDCK cell monolayer

The MDCK cells were grown in 75 cm^2 corning flasks in DMEM supplemented with 10% (v/v) fetal bovine serum and 1% penicillin-streptomycin solution at 37 °C with 5% CO_2 and 95% air with high humidity. The cells were harvested by trypsin-EDTA solution once they reached 80%–90% confluency, and then seeded on 24 Transwell® inserts (1 μm pore size, 0.33 cm^2 growth area) at 6.3×10^4 cells/cm^2 with 0.3 mL growth media on the apical side. 1 mL growth media were added on the basolateral side. The growth media was changed every other day and TEER value was measured. The transport was run on Day 7 once the monolayer had been formed and the TEER value had reached above 300 Ω·cm^2. The monolayer was washed twice with HBSS and incubated in HBSS for 30 min. Then the transport media on the apical side were replaced by 0.3 mL of unlabeled insulin or FITC-insulin (2 mg/mL in HBSS). Samples of 200 μL each were withdrawn from the basolateral side at the predetermined time points until 3 h. After each withdrawal,

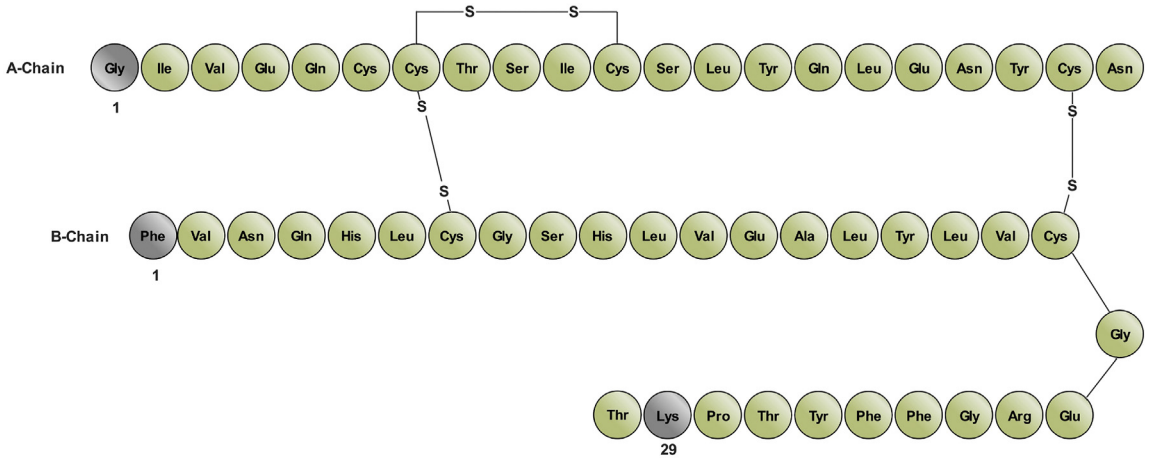

Fig. 1. Primary structure of human insulin with grey colored circles indicating primary amine sites for FITC labeling.

the same volume of fresh HBSS was added to the basolateral side. The samples containing FITC-insulin were analyzed for fluorescence by Promega GloMax DISCOVER plate reader (excitation filter: Blue 475 nm, emission filter: 500–550 nm) and unlabeled insulin was analyzed by ELISA.

3. Results and discussion

3.1. FITC labeling of human insulin

Human insulin has 51 amino acids divided into two chains: Chain A containing 21 amino acids and Chain B containing 30 amino acids (Fig. 1). There are three primary amine groups in an insulin molecule. Bromer et al. [11] reported that FITC could bind to the three primary amine sites and form mono-, di- and tri-conjugated FITC-insulin. The order of reactivity of the three primary amines has been found to be B1 (Phe) > A1 (Gly) > B29 (Lys) [11].

In the present study, the labeling reaction was conducted at different molar ratios of FITC to insulin and different reaction time to investigate the impact of these two factors on the products (mono-, di- and tri-conjugated FITC-insulin). The reaction mixture after the gel filtration was analyzed by HPLC connected with an absorbance detector (VWD) and a fluorescence detector (FLD). FITC-insulin gave peaks in both the absorbance and fluorescence chromatograms at around the same retention time, whereas the unlabeled insulin showed a peak only in the absorbance chromatogram but not in the fluorescence chromatogram. Among the 32 fractions from the gel filtration, fractions 4–9 had the labeled insulin, showing peaks in both chromatograms, and they were

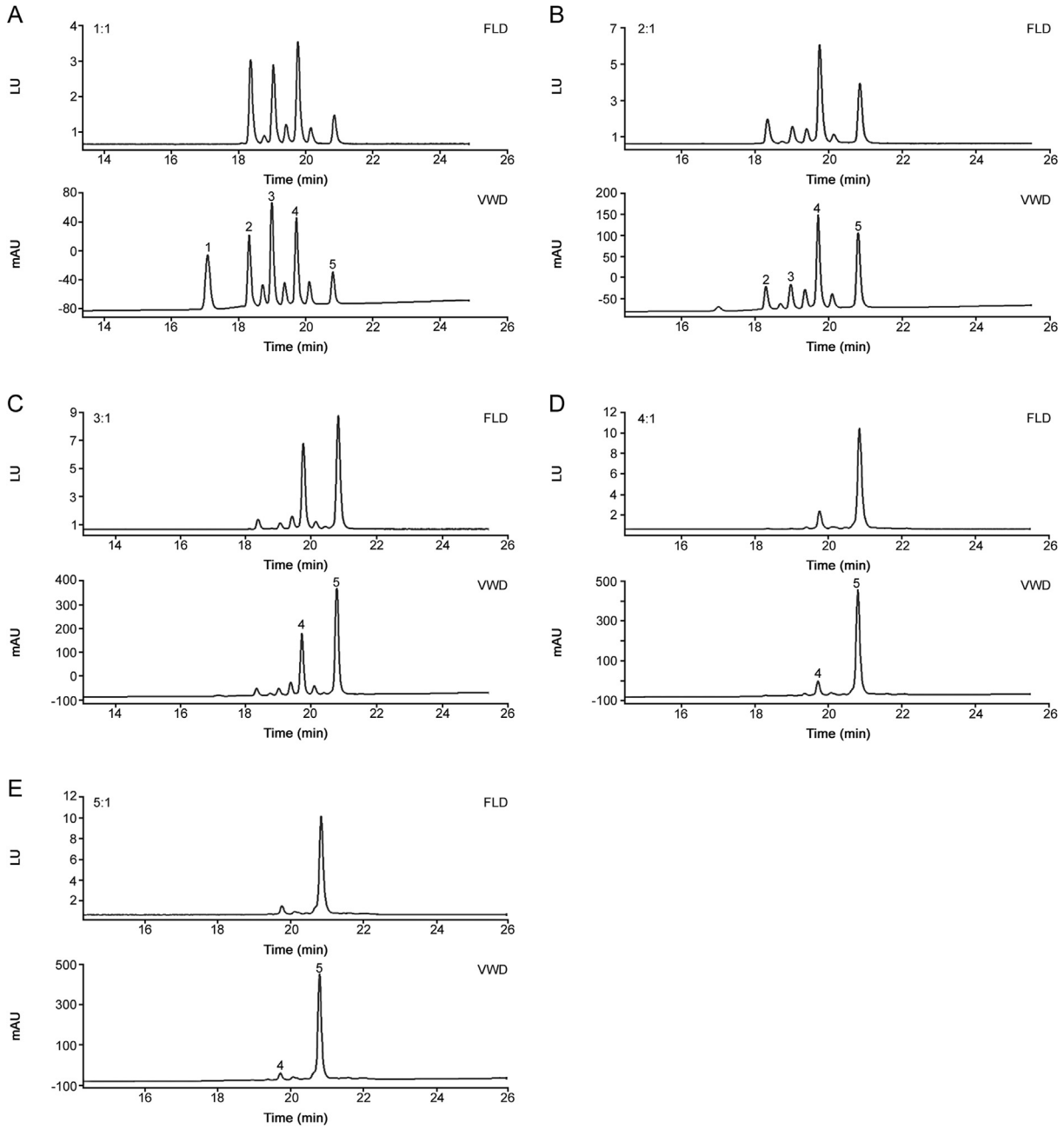

Fig. 2. Chromatograms of the FITC-labeled human insulin, with 150 min reaction time and FITC:insulin molar ratio of (A) 1:1, (B) 2:1, (C) 3:1, (D) 4:1 and (E) 5:1, respectively.

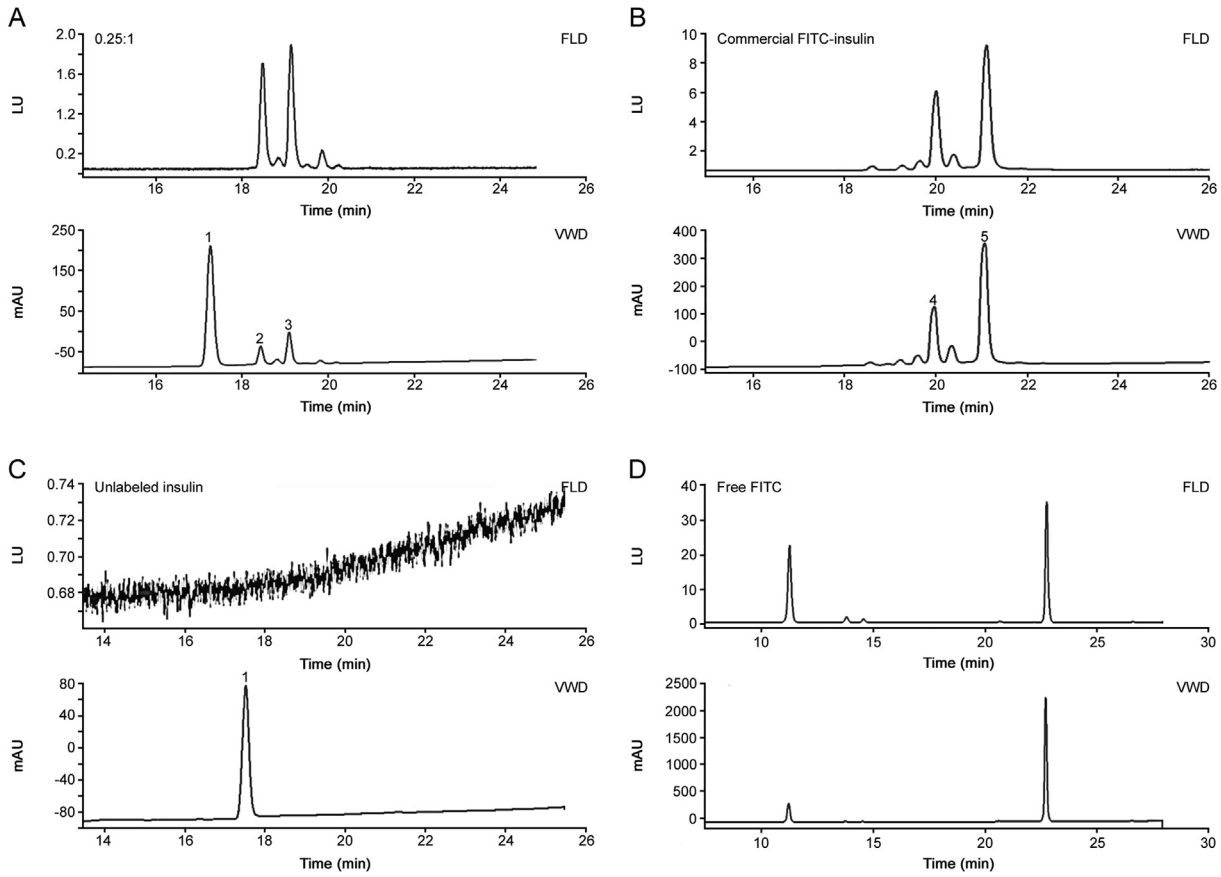

Fig. 3. Chromatograms of (A) 0.25:1 molar ratio (90 min reaction time) FITC-insulin; (B) Commercial FITC-insulin; (C) Unlabeled insulin; (D) Free FITC.

combined. Fig. 2 and Fig. 3A represent the chromatograms of the combined fractions 4–9 from each reaction as compared to the unreacted insulin (Fig. 3C), the unreacted FITC (Fig. 3D) and the commercial FITC-insulin (Fig. 3B). RP-HPLC assay of FITC-insulin is a well-established method to identify different FITC-insulin conjugates [4,6]. Although the retention time may vary, the elution pattern for FITC-insulin conjugates remains the same. Based on the previous reports [4,6], the peaks in the absorbance chromatograms were identified as follows: Peak 1 – unlabeled insulin; Peak 2 – mono-conjugated FITC-insulin at B1; Peak 3 – mono-conjugated FITC-insulin at A1; Peak 4 – di-conjugated FITC-insulin at B1 and A1; Peak 5 – tri-conjugated FITC-insulin at B1, A1 and B29. Small peaks in between Peak 2 and Peak 5 have also been shown in the commercial FITC-insulin chromatogram (Fig. 3B) as well as the FITC-insulin chromatograms previously reported by Hentz et al. [6]. These small peaks could be due to impurities. Further purification of gel filtered fractions is needed to produce FITC-insulin conjugates at stoichiometric purity. Peaks 1 to 5 in absorbance chromatogram can be characterized by MALDI-TOF mass spectrometry and top-down analysis method for branched proteins by mass spectrometry [12–14].

When reaction was carried out for 150 min, different molar ratios of FITC:insulin produced different conjugates which showed peaks in both detectors as follows (Fig. 2): (A) 1:1 molar ratio had unlabeled insulin and it produced mono- and di-conjugates with little quantity of tri-conjugates. (B) 2:1 molar ratio produced di- and tri-conjugates where di-conjugate was determined to be in higher amount than tri-conjugate. (C) 3:1 molar ratio showed both di-and tri-conjugates where tri-conjugate was determined to be in higher amount than di-conjugate. (D and E) Molar ratios 4:1 and 5:1 produced tri-conjugate with very little amount of di-conjugates.

Molar ratio 0.25:1 with 90 min of reaction time gave mono-conjugated FITC-insulin along with unlabeled insulin (Fig. 3A). The chromatograms of the products from molar ratio 3:1 FITC-insulin were similar as that of the commercial FITC-insulin (Figs. 2C and 3B). Increasing the reaction time for 4:1 to 240 min did not give pure tri-conjugated FITC-insulin. The products still had little amount of di-conjugates. Unbound FITC gave peaks at 11 min and 22 min (Fig. 3D) which further confirmed the absence of unbound FITC in the labeled insulin fractions. Mixture of di- & tri-conjugate (Fig. 2C) which is similar to commercially available FITC-insulin and tri-conjugate with little amount of di-conjugates (Fig. 2D) was synthesized in less than 4 h whereas previously published method by Jacob et al. and Hentz et al. [4,6] takes 20 h or more to synthesize different FITC-insulin conjugates.

3.2. Lipophilicity and hydrophilicity of FITC-insulin

In order to evaluate the lipophilicity and hydrophilicity of the FITC-insulin, the partition coefficient (P) of the unlabeled insulin, mono- and tri-labeled insulin in the octanol/water system was determined. P value of the unlabeled insulin, mono-conjugate and tri-conjugate was determined to be 0.07 ± 0.04, 0.08 ± 0.04 and 0.12 ± 0.02, respectively (Fig. 4).

3.3. Transport of FITC-insulin through MDCK cell monolayer

MDCK cells have been used as a model cellular barrier for determining the intestinal epithelial drug transport for many years

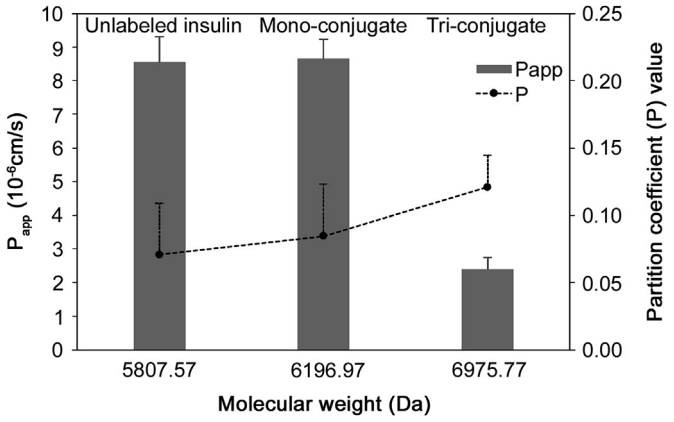

Fig. 4. Permeability coefficient (P_{app}), molecular weight and partition coefficient (P) value of unlabeled insulin, mono- and tri-conjugates (Mean ± SD, $n = 3–6$).

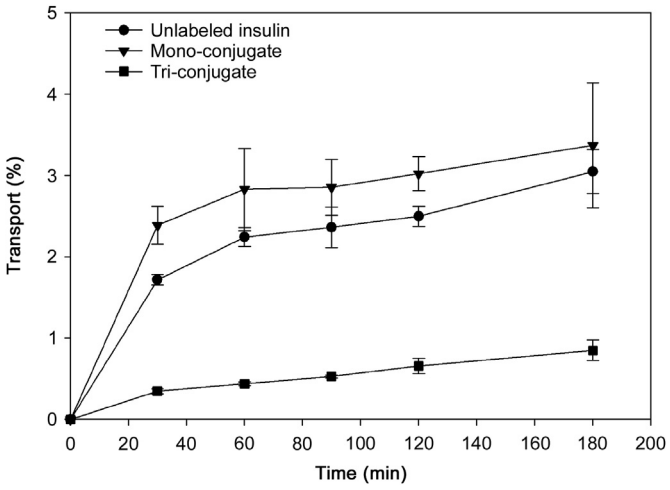

Fig. 5. Transport of mono- and tri-conjugated FITC-insulin through MDCK cell monolayer (Mean ± SD, $n = 6$).

[15], and take comparatively short time to grow and form tight junctions when grown on semi-permeable membranes. Therefore, MDCK cell monolayer was chosen for this study to determine the effect of FITC labeling of insulin on its transport. The mono-conjugated FITC-insulin (22%) with unlabeled insulin prepared from 0.25:1 molar ratio (Fig. 3A), tri-conjugated FITC-insulin (84%) with little amount of di-conjugate prepared from 4:1 molar ratio (Fig. 2D) and the unlabeled insulin were chosen for the transport study. The percentage amount of mono-conjugate and tri-conjugate was calculated through chromatogram integration. Transport samples of mono-conjugated FITC-insulin and tri-conjugated FITC-insulin were analyzed by plate reader for fluorescence measurement. Therefore, presence of unlabeled insulin together with mono-conjugated FITC-insulin did not have any interference with the transport results. The results are presented in Fig. 4 and Fig. 5.

The unlabeled insulin and mono-conjugated FITC-insulin showed significantly higher ($p < 0.05$) transport than tri-conjugated FITC-insulin (Fig. 5). It has been very well known that lipophilicity and molecular weight play major roles in passive diffusion of drug molecules through biological membranes [16]. It is known that with the increase of molecular weight, the diffusion (permeability) decreases, and with the increase of lipophilicity, the permeability increases. For lower molecular weight compounds (MW 200–400), the lipophilicity shows a linear relationship with permeability. But for higher molecular weight compounds (MW > 500), lipophilicity does not have linear relationship with permeability, and molecular weight has a significant impact on permeability [16,17]. Even though, the partition coefficient value of tri-conjugated FITC-insulin is higher than mono-conjugate and unlabeled insulin, it showed decreased transport which could be due to the increase in molecular weight (Fig. 4). Increase in molecular weight of tri-conjugate was observed to be due to attachment of three FITC molecules to insulin molecule as compared to mono-conjugate which had only one FITC molecule attached to one insulin molecule. Low purity of the conjugates prepared could possibly complicate the interpretation of the results.

FITC is much more hydrophobic (log P = 5.25) than insulin [18]. Mono-conjugated FITC-insulin showed slightly higher but not significantly different transport than the unlabeled insulin. Slightly increased permeation could be due to the lipophilicity induced by FITC molecule. Increase in molecular weight of insulin conjugate could be the reason for decreased permeability of tri-conjugated FITC-insulin through MDCK cell monolayer.

4. Conclusion

A quick, reliable and relatively simple method was developed to synthesize the different conjugates of FITC-insulin. Extent of conjugation of FITC to insulin did affect the permeability of insulin across cell monolayer.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpha.2019.08.002.

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