Supplementary Material

QbD-based fabrication of transferrin-anchored nanocarriers for targeted drug delivery to macrophages and colon cells for mucosal inflammation healing

Mahira Zeeshan[1,4], Qurat Ul Ain[1], Ahad Sunny[1], Faisal Raza[2], Muhammad Mohsin[3], Salman Khan[1], Benno Weigmann[4], Hussain Ali[1]*

[1]Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan

[2]School of Pharmacy, Shanghai Jiao Tong University, Shanghai, 200240, China

[3]Riphah Institute of Pharmaceutical Sciences, Islamabad, Pakistan

[4]Department of Medicine 1, University of Erlangen-Nuremberg, Kussmaul Campus for Medical Research, Erlangen 91052, Germany

*Correspondence at: h.ali@qau.edu.pk, Phone No. + 925190644021; +923471919606, Address: Department of Pharmacy, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad 45320, Pakistan

1. Methods

1.1. HPLC method

HPLC analysis of Tofa was performed through the isocratic method using reversed-phase Agilent HPLC (Agilent technologies 1200 series). The mobile phase consisting of 10mM ammonium acetate (pH 5) and acetonitrile (70:30, v/v), flows at a rate of 1.0 mL/minute. Tofa stock solution was prepared in the mobile phase at a concentration of 1mg/mL, then various dilutions were prepared in the range of 1.5625-100 µg/mL for a standard curve. The volume of injection for the standard or samples was 20 µL and HPLC was assisted with a UV detector that operates at a wavelength of 287 nm. C18 reverse-phase column (250 × 4.6 mm, 5 µm pore size; supelcosil LC-1, Supelco) was used for chromatographic separation of Tofa, which was maintained at a temperature of 35°C. The method proved to be linear with an R^2 value of 0.9994 and obtained a retention time of 9.035 minutes.

Another HPLC method was developed for the quantification of Tofa using reversed phased Agilent HPLC (Agilent 1260 Infinity II series), assisted with C18 Phenomenex Luna column (150 mm × 4.6 mm, 0.3 um pore size), maintained at 30°C. The mobile phase was constituted with Millipore water and Acetonitrile (70:30). The method was isocratic, with similar conditions. The calibration curve was obtained at a concentration of 0.78125-100 µg/mL with a retention time of 5-5.19 min ($R^2 = 0.9998$).

1.2. Selection of an appropriate method for Tofa-P/tfr NCs formation

To prepare Tofa-P/tfr NCs with good stability, particle size, and tfr adsorption, the following methods were tried:

i. Standard method of tfr adsorption on PLGA NCs

This method is the standard method to prepare PLGA/tfr nanocarriers [1]. According to this method, blank PLGA nanocarriers were developed by a simple O/W emulsion method. Once PLGA nanosuspension was centrifuged (13500 rpm, 35 minutes) and pellet of purified nanocarriers was obtained, then it was re-dispersed in tfr-HEPES solution (1 mg/mL) and stirred overnight for the adsorption of tfr. The next day, PLGA/tfr nanosuspension was washed and freeze-dried.

ii. Tfr adsorption during emulsification

The standard method was modified. In this method, PLGA was dissolved in organic solvent and then added dropwise into the aqueous solution comprising 2% PVA solution and tfr-HEPES solution. Then probe sonicated to emulsify the two phases and kept stirred for 4-8 hours. Afterward, solvent was evaporated overnight with stirring. Therefore, tfr adsorbed to the PLGA surface during the formation of nano-emulsion.

iii. Chemical conjugation of tfr to PLGA NCs

At first PLGA NCs were prepared, and then dispersed in PBS (pH 5.5, 100mM) containing EDC/NHS (400mM/ 100mM). The reaction was conducted for 1 hour in the dark at 15°C. Afterward, spin it (13000 rpm, 30 min) thrice to obtain a purified pellet. The activated nanocarrier pellet was added to PBS (pH 7.4) containing tfr (1 mg) and stirred for 8 hours for

the conjugation of tfr to the nanocarriers. Then centrifuged and freeze-dried to get purified PLGA/tfr NCs.

1.3. 3^3 Box-Behnken DOE to prepare Tofa-P/tfr NCs

Supp. Table 1: Studied independent variables at three different levels (-1, 0, +1) in 3^3 Box-Behnken DOE to prepare Tofa-P/tfr NCs of desired characteristics

Studied variables at 3 levels, optimized through 3^3 Box-Behnken design			
Independent variables	**Coded levels**		
	Low (-1)	**Medium (0)**	**High (+1)**
A: Tfr conc. (mg)	0.50	0.75	1.00
B: Drug conc. (mg)	0.50	1.00	2.00
C: Surfactant conc. (%)	1.00	2.00	3.00
Response variables	**Desired criteria**		
X: Particle size (nm)	Minimum		
Y: Drug entrapment (%)	Maximum		
Z: Tfr adsorption (mg)	Maximum		

1.4. Physicochemical characterization and protein (tfr) content determination

Encapsulation efficiency (EE) was determined from the free amount of drug in the supernatant collected during the washing of nanocarriers using the HPLC method at 287 nm. Further, drug content loaded into the nanocarrier was estimated by dispersing freeze-dried nanocarriers in acetone-methanol (1:1) solution for the dissolution of excipients and polymers for 4 hours, then evaporated to a residue. Afterward, acetonitrile: Millipore water mixture (3:7) was added to extract the drug overnight into the solvent system, then centrifuged to remove the residue, and the collected supernatant was analyzed under HPLC for drug quantification. The formulas used to determine EE and drug content are mentioned in Equations 1 and 2, respectively.

$$\% EE = \frac{Total\ conc.\ of\ drug - Free\ drug\ conc.}{Total\ conc.\ of\ drug} \times 100 \quad \textbf{.....1}$$

$$Drug\ content\ (\mu g/mg) = \frac{Drug\ conc.\ extracted\ from\ nanocarriers}{Total\ amount\ of\ polymer + excipients} \quad \textbf{..... 2}$$

Tfr protein content was determined through the Bradford assay and a Nanodrop instrument named as Titertek-Berthold Colibri Microvolume Spectrometer (Berthold Detection Systems GmbH, Germany). Nanodrop measured the adsorption of tfr onto the nanocarriers through quantification of free tfr in the supernatant of the blank nanocarriers at 280/260 nm. Then the amount of adsorbed tfr was calculated using Equation 3.

$$Conc.\ of\ tfr\ adsorbed = \frac{total\ conc.\ of\ tfr - free\ tfr\ content}{total\ conc.\ of\ tfr} \quad \text{.....3}$$

Further, tfr or BSA protein content was assessed through the Bradford assay [2]. To prepare a standard curve, BSA was dissolved in distilled water at various concentrations (7.8125-1000 µg/mL), and 100 µL from each BSA dilution was mixed with Bradford reagent (5 mL, 5X) and incubated for 5-10 minutes and analyzed at 595 nm. For samples, unbound tfr in the supernatants of the centrifuged nanocarriers were collected (100 µL) and mixed with Bradford reagent (5 mL) separately, followed by incubation for 5-10 minutes. Then the standards and samples were analyzed under a spectrophotometer at 595 nm.

1.5. Hemolysis study-blood withdrawal criteria

The blood was withdrawn from young, healthy volunteers with informed consent and the experiments were performed according to the standard WHO blood withdrawing protocols [3]. Following criteria were considered for blood withdrawing:

- Healthy individual, weight above 60 kg, Hb > 13.5, non-pregnant, normal blood pressure, no blood-borne disease, no communicable disease, normal body temperature.
- Secondly, the amount of blood withdrawn was just 3 ml per 60 kg individual once, which is far less than the standard IRB protocol (i.e., 5 ml/kg in 24 hours), therefore, does not required special IRB approval.

1.6. qRT-PCR analysis of expression of proteins

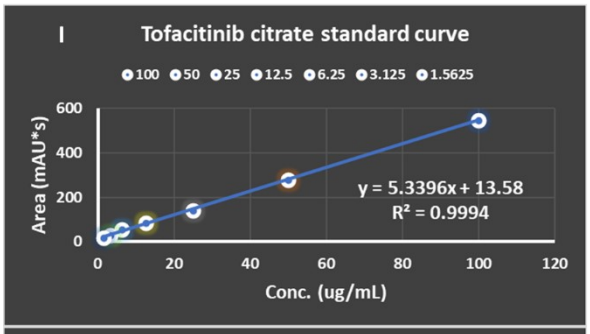
Supp. Table 2: Forward and reverse primer sequence of the targeted genes

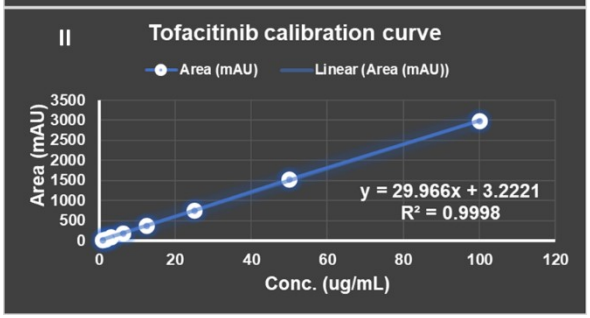
Gene	Forward primer	Reverse primer
STAT-1	CTGAATATTTCCCTCCTGGG	TCCCGTACAGATGTCCATGAT
TFR-1	GGTAAACTGGTCCATGCTAAT	CCCTGCTCTAACAATCACTAAA
iNOS	GGCAGCCTGTGAGACCTTTG	GCATTGGAAGTGAAGCGTTTC
E-Cadherin	GGCGCCACCTCGAGAGA	TGTCGACCGGTGCAATCT T
β-actin	CGGTGGACATTGGTTCTGG	CTGAGGAAGGGCAGAAGTTC

2. Results

2.1. HPLC Calibration Curve

Supp. Figure 1: Tofacitinib citrate (Tofa) HPLC calibration curves (I-II)





HPLC Conditions

- **Mobile phase: 10mM monoammonium phospahte (pH 5): Acetonitrile (70:30)**
- **Isocratic method**
- **Wavelength: 287 nm**
- **Flow rate: 1mL/min**
- **Injection volume: 20uL**
- **Retention time: 9.035 min**
- **Stock solution: 100 ug/ml in mobile phase**
- **Column temperature: 35°C**

- **Mobile phase: Millipore water and Acetonitrile (70:30)**
- **Isocratic method**
- **Wavelength: 287 nm**
- **Flow rate: 1mL/min**
- **Injection volume: 20uL**
- **Retention time: 5.-5.19 min**
- **Stock solution: 100ug/ml in mobile phase**
- **Column temperature: 30°C**
- **column: C18 phenomenax 150mmx4.6mm, 0.3u pore size**
- **HPLC: Agilent 1260 Infinity II**

2.2. Selection of an appropriate method for Tofa-P/tfr NCs formation

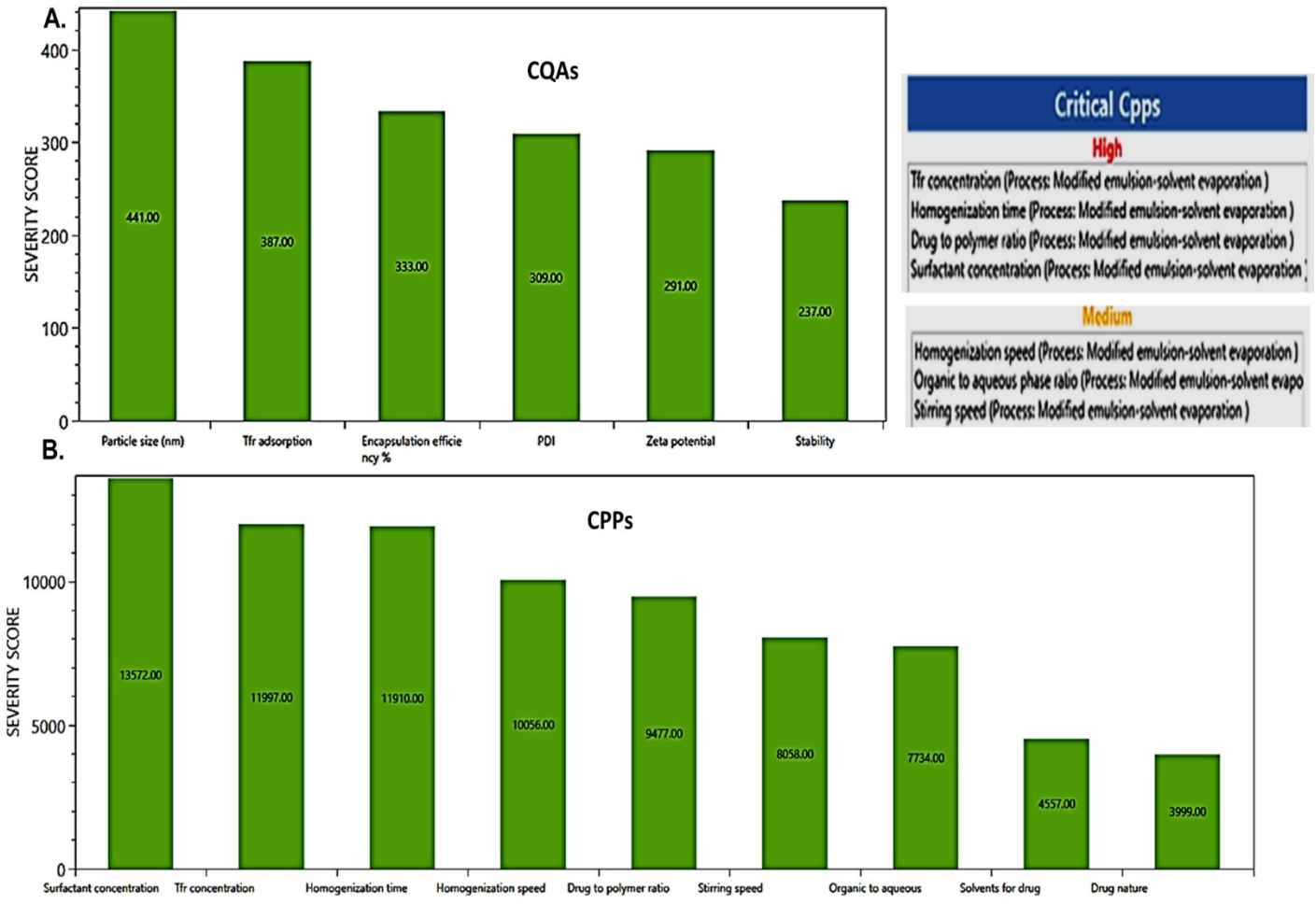
All three methods formed stable nano-suspension with good particle size (<200 nm). However, method ii was selected because of maximum tfr adsorption from a relatively simple and cost-effective method. In comparison to method **i**, this method had relatively more tfr adsorption and decreased number of steps. When compared to chemical conjugation, this method is more safer

and environment friendly with no use of toxic materials like EDC and NHS. Further, the tfr adsorption was good. All three methods were also tested to prepare drug-loaded P/tfr NCs. The tedious method of chemical conjugation always resulted in lesser drug entrapment efficiency.

Together with these considerations, tfr physical adsorption during emulsification (method ii) was selected for further optimization.

2.3. QbD screening of influential CQAs and CPPs with severity scores

Supp. Figure 2: Screening of critical quality attributes (A), and critical process parameters (B) through risk-based assessment of severity scores



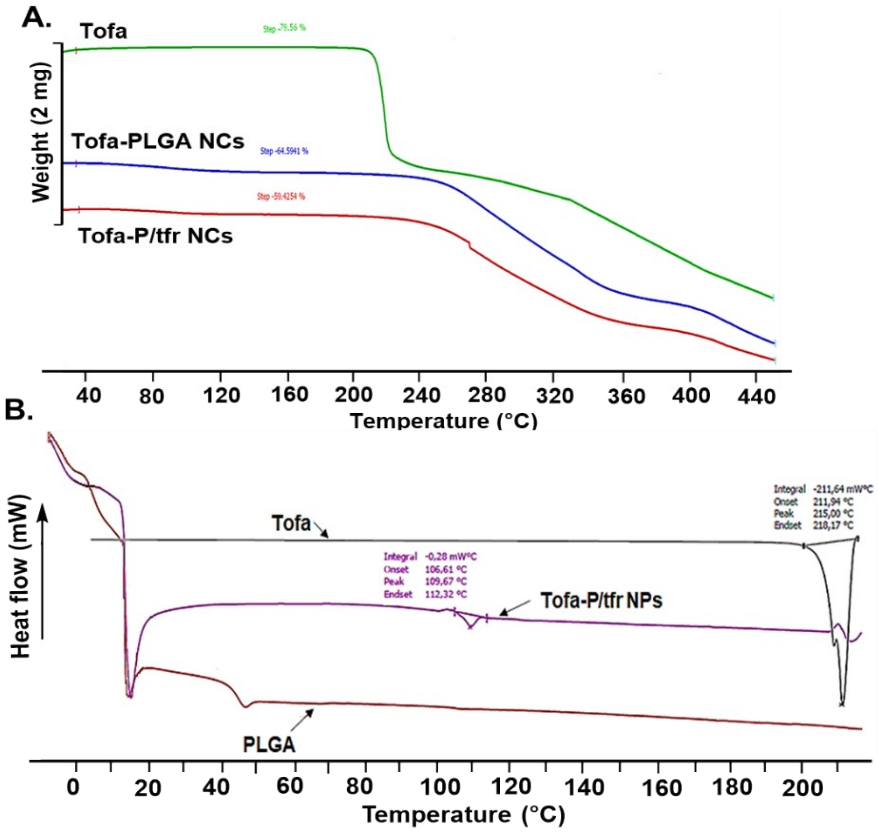
2.4. DSC, TGA and ATR-FTIR analysis

TGA analysis of drug, Tofa-PLGA NCs, and Tofa-P/tfr NCs revealed their degradative patterns at rising temperatures. The TG graph depicted a sharp degradation peak of the drug at 230-240°C. Tofa melts at around the same temperature [4]. It is followed by a shallow decline till the end. While the patterns of Tofa-PLGA NPs and Tofa-P/tfr are alike and have a slight shift after 400°C; about 66% and 59% of the nanocarriers are consumed till 450°C, respectively (**Supp. Figure 3A**).

The DSC thermogram of Tofa exhibited a sharp endotherm at 215°C, representing its melting point because of crystalline content (**Supp. Figure 3B**). While the thermograms of PLGA polymer and Tofa-P/tfr NCs were devoid of any sharp endotherm. The flattened curve of the nanocarrier indicated that Tofa entrapped within the Tofa-P/tfr NCs in the amorphous form rather than crystalline. Only a small endotherm at 109°C was prominent in Tofa-P/tfr NPs, which might be representative of tfr adsorbed on the surface [5]. A starting downward curvature (~10-15°C) was due to an abrupt change in temperature from freezing to 10°C (**Supp. Figure 3B**).

The ATR-FTIR spectra of drug, polymer, and nanocarriers are represented in **Figure 5E**. The spectrum of PLGA consisting of peaks at 3470 cm-1 (-OH), 2998 cm-1 and 2952 cm-1 (-CH2), 1742 cm-1 (C=O), 1422 cm-1 (C-OH in-plane), 1381 cm-1 (-CH3), 1161 cm-1 (ester C-O), and 1082 cm-1 (alcoholic C-O) (**Figure 5E**). The drug Tofa had major FTIR peaks at 3375 cm-1, 3129 cm-1, 1731 cm-1, 1615 cm-1, 1340 cm-1, 1207 cm-1 and 842 cm-1 corresponding to -NH stretching band, C=C, C=O, secondary C=C stretch, C-N, C-O stretch, and -C-H respectively (**Figure 5E**). FTIR spectrum of tfr had an amine (-NH-) stretch at 3278 cm-1, with an amide band at 1638 cm-1, similar to the literature report (42). The other peaks are visible at 2961 cm-1 (-CH2), 2187 cm-1 (C≡N), 1507 cm-1 (CH2/CH3), and 1386 cm-1 (CH), 1235 cm-1 and 1071 cm-1 (C-O) (**Figure 5E**).

Supp. Figure 3: Physicochemical characterization of Tofa-P/tfr NCs: (A) TGA thermograms, (B) DSC thermogram of nanocarriers and the drug



2.5. Stability studies

Supp. Table 3: Effect of storage conditions on freeze-dried Tofa-P/tfr NCs (mean ± SD, n=3)

Lyophilized Tofa-P/tfr NCs	Initial		After 6 months	
	4°C	25°C	4°C	25°C
Particle size (nm)	208 ± 1.36	208 ± 1.36	210.5 ± 3.87	216.6 ± 1.02
Zeta potential (mV)	-8.64 ± 1.73	-8.64 ± 1.74	-8.1 ± 0.53	-7.97 ± 2.89
PDI	0.121±0.004	0.121±0.004	0.121± 1.25	0.136± 1.24
EE%	80.97 ± 0.79	80.97 ± 0.80	77.92 ± 2.59	76.92 ± 2.27
tfr adsorption	0.886 ± 0.015 (88.6%)	0.886 ± 0.015 (88.6%)	0.79 ± 0.15 (79%)	0.71 ± 0.23 (71%)

2.6. RFT normal values (as per lab test)

Renal Function Tests		
Serum Urea	mg/dL	12 - 45
Serum Creatinine	mg/dL	Upto 1.20
Serum Uric Acid	mg/dL	Males 2 - 7 Females 2 - 6

2.7. C-Reactive Protein (CRP) values

Test Name	Unit	Reference Range
CRP (Quantitative)	mg/L	Negative : Upto 5.9 Positive : > 6.0

REFERENCE

[1] M.F. Frasco, G.M. Almeida, F. Santos-Silva, M.d.C. Pereira, M.A. Coelho, Transferrin surface-modified PLGA nanoparticles-mediated delivery of a proteasome inhibitor to human pancreatic cancer cells, Journal of Biomedical Materials Research Part A 103(4) (2015) 1476-1484.

[2] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Analytical Biochemistry 72(1) (1976) 248-254.

[3] W.H. Organization, WHO guidelines on drawing blood: best practices in phlebotomy, World Health Organization2010.

[4] S. Bashir, M. Aamir, R.M. Sarfaraz, Z. Hussain, M.U. Sarwer, A. Mahmood, M.R. Akram, M.N. Qaisar, Fabrication, characterization and in vitro release kinetics of tofacitinib-encapsulated polymeric nanoparticles: A promising implication in the treatment of rheumatoid arthritis, International Journal of Polymeric Materials and Polymeric Biomaterials 70(7) (2021) 449-458.

[5] S. Jose, C.T. A, R. Sebastian, S.M. H, A.N. A, A. Durazzo, M. Lucarini, A. Santini, E.B. Souto, Transferrin-Conjugated Docetaxel-PLGA Nanoparticles for Tumor Targeting: Influence on MCF-7 Cell Cycle, Polymers (Basel) 11(11) (2019) 1905.