**QUEEN'S
UNIVERSITY
BELFAST**

A Difunctional Pluronic®127 Based Insitu Formed Injectable Thermogels as Prolonged and Controlled Curcumin Depot, Fabrication, Invitro Characterization and Invivo Safety Evaluation

Khan, S., Akhtar, N., Usman Minhas, M., Shah, H., Khan, K. U., & Thakur, R. R. S. (2020). A Difunctional Pluronic®127 Based Insitu Formed Injectable Thermogels as Prolonged and Controlled Curcumin Depot, Fabrication, Invitro Characterization and Invivo Safety Evaluation. *Journal of Biomaterials Science, Polymer Edition*. <https://doi.org/10.1080/09205063.2020.1829324>

Published in:

Journal of Biomaterials Science, Polymer Edition

Document Version:

Peer reviewed version

Queen's University Belfast - Research Portal:

[Link to publication record in Queen's University Belfast Research Portal](#)

General rights

Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

A Difunctional Pluronic®127 Based *Insitu* Formed Injectable Thermogels as Prolonged and Controlled Curcumin Depot, Fabrication, *In vitro* Characterization and *In vivo* Safety Evaluation

Samiullah Khan, Naveed Akhtar, Muhammad Usman Minhas, Hassan Shah, Kifayat ullah Khan & Raghu Raj Singh Thakur

To cite this article: Samiullah Khan, Naveed Akhtar, Muhammad Usman Minhas, Hassan Shah, Kifayat ullah Khan & Raghu Raj Singh Thakur (2020): A Difunctional Pluronic®127 Based *Insitu* Formed Injectable Thermogels as Prolonged and Controlled Curcumin Depot, Fabrication, *In vitro* Characterization and *In vivo* Safety Evaluation, Journal of Biomaterials Science, Polymer Edition, DOI: [10.1080/09205063.2020.1829324](https://doi.org/10.1080/09205063.2020.1829324)

To link to this article: <https://doi.org/10.1080/09205063.2020.1829324>

Accepted author version posted online: 25 Sep 2020.

Submit your article to this journal [↗](#)

View related articles [↗](#)

View Crossmark data [↗](#)

A Difunctional Pluronic®127 Based *Insitu* Formed Injectable Thermogels as Prolonged and Controlled Curcumin Depot, Fabrication, *Invitro* Characterization and *Invivo* Safety Evaluation

Samiullah Khan^{1,*}, Naveed Akhtar², Muhammad Usman Minhas³, Hassan Shah², Kifayat ullah Khan², Raghu Raj Singh Thakur⁴

¹Department of Pharmacy, The University of Lahore Gujrat Campus, Pakistan

²Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur 63100, Punjab, Pakistan

³College of Pharmacy, University of Sargodha, Sargodha Pakistan.

⁴School of Pharmacy, Queen's University Belfast, 97 Lisburn Road, Belfast BT9 7BL, United Kingdom

* **Corresponding author** Dr. Samiullah Khan, Department of Pharmacy, The University of Lahore Gujrat Campus, Pakistan. Email: sami_pharmacist99@hotmail.com

Abstract

Curcumin has been reported to be used widely against many types of pathological conditions in clinics. However due to its limitations such as poor solubility, poor oral absorption and low stability has limited its applications. In current study, a series of novel chemically cross-linkable depot gel formulations were developed based on thermoresponsive micellar polymer (Pluronic® 127) with polyelectrolyte hydrophilic monomer i.e. 2-acrylamido-2-methylpropane sulfonic acid by cold and *insitu* grafting polymerization method. The formulations were aimed to deliver curcumin at controlled rate from *insitu* formed depot after administration through subcutaneous route *invivo*. The sol-gel phase transitions of formulations were observed by rheological analysis, tube titling and optical transmittance measurements. Maximum swelling of gel formulations was observed at pH 7.4 and below CGT i.e. 25°C. The *invitro* release profile exhibit maximum drug release at pH 7.4 and 25°C owing to relaxed gel state. *Invitro* degradation profile of gel formulations showed controlled degradation rate. Cells growth inhibition study confirmed the biocompatibility and safe nature of bare gel formulations against L929 cell lines. *Invitro* cytotoxic study showed that curcumin loaded in gel formulation has controlled pharmacological activity against HeLa and MCF-7 cancer cells as compared to free drug solution. The IC₅₀ values calculated for pure curcumin solution (30±0.77 µg/ml for HeLa and 27±0.39 µg/ml for MCF-7) were found higher in comparison to curcumin loaded thermogels; against HeLa (19±0.28 µg/ml and 23±0.81 µg/ml) and MCF-7 (22±0.54 µg/ml and 21±0.49 µg/ml). Histopathological and Haematological analysis showed the biocompatible nature of hydrogels. Structural confirmation was done by Fourier transformed infra-red spectroscopy (FTIR) and proton nuclear magnetic resonance spectroscopy (¹HNMR). Differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) confirmed the thermal stability of the gel formulation. The porous structure of gel formulations was assessed by scanning electron microscopic (SEM) analysis. Results concluded that newly developed gel formulations has thermoresponsive behavior with phase transition at body temperature and can be used as *insitu* controlled drug depot.

Graphical abstract

Key Words: Thermoresponsive depot; Pluronic®127; Poly (2-acrylamido-2-methylpropane sulfonic acid), MTT assay, Histopathology

1. Introduction

Curcumin, a natural yellow active component extracted from the rhizome of curcuma (*Curcuma Longa*, Zingiberaceae) has been well known for many pharmacological activities such as antitumor, antibacterial, anti-inflammatory, antioxidant and liver protection. Its broad spectrum anticancer activity has been confirmed against many cancer cell lines in *invitro* cell culture experiments and *in vivo* in animals. However the applications of curcumin as an anticancer agent have been hampered due to its insolubility in water, poor bioavailability and low stability in alkaline media [1, 2]. Currently many curcumin formulations include granules and tablets. Some chitosan based nanogels and microparticles have also been reported for curcumin delivery whose stability is still not good [3–6]. Moreover orally taken curcumin is rapidly metabolized in gastrointestinal tract lead to decreased blood circulation that need the reform of the formulation. Oftenly the uniform dispersal of the hydrophobic drug molecules in drug delivery carriers is thermodynamically unstable and demands the development of stable delivery devices.

Hydrogels are versatile soft fascinating materials with three-dimensional (3D) cross-linked networks. They have the availability to absorb water and biological fluids without dissolving and maintain their three dimensional integrity [7]. Over the last few decades, the stimuli-responsive hydrogels has gained huge attention owing to their sensitivity to the environmental stimulus, including changes in pH, temperature, electric field, magnetic field and chemical modifications [8].

Water solutions of hydrophobically modified hydrophilic polymers refers a class of soft materials exhibiting remarkable rheological characteristics. Such polymers which exist in solution state below

certain temperature display high viscosity producing semi-solid hydrogel as the drug depot upon injection at body temperature [9]. The temperature at which this sol-gel phase transition occur refers to critical gelation temperature (CGT), while such polymers are called thermogelling polymers [10]. Injectable hydrogels formed *in situ* are of great interest for drug delivery as they require injection for *in vivo* depot formation and avoids the requirement for surgical implantation as needed for prefabricated sustained release implants [11].

Generally for *insitu* injectable hydrogels synthesis, physical supramolecular or chemical grafting approaches have been used [12]. Physically cross-linked hydrogels utilize the self-assembly of variety of functional molecules through weak interaction forces such as host/guest inclusion complex, hydrogen bonding, hydrophobic interactions, ionic, stereocomplex formation, electrostatic interactions and π - π staking [13]. Chemically cross-linked gels which are termed as permanent/irreversible gels are formed by the covalent grafting between the functional monomer or polymer chains. This chemical grafting is created with the help of crosslinking agents e.g. glutaraldehyde, epoxy compounds, dialdehyde and formaldehyde [14].

In the development of *insitu* forming hydrogels, various synthetic thermo-responsive polymers such as poly (N-isopropylacrylamide) [15], vinylcaprolactam [16], poly (ethylene oxide) (PEO)/poly (propylene oxide) (PPO) (PEO-PPO-PEO) tri-block copolymers (Pluronics) [17, 18], polyphosphazenes [19, 20] and PEO/poly (D, L-lactide-co-glycolide) (PLGA) tri-block copolymers [21] that display thermo-sensitive property have been widely studied.

Triblock copolymer (commercially called Pluronic[®], also named poloxamer) consisting of series of poly (ethylene oxide)-poly (propylene oxide)-poly (ethylene oxide) (PEO-PPO-PEO) can induce sol-gel transition as a result of hydration and dehydration of polymer chains in aqueous solutions in response to decrease and increase in temperature respectively. These tri-block copolymers produce spherical micelles in aqueous solution *via* hydrophobic interaction among the middle PPO segments [22]. At specific polymer concentration, the PPO hydrophobic groups in these associative polymers take part in intermolecular entanglements that act as breakable crosslinking groups creating a transient 3D polymer network [17].

In Pluronic[®] family, Pluronic F127 (PF-127) also known as Poloxamer 407 is the most widely studied member owing to its good water solubility and comparatively longer PPO block involved in hydrophobic interaction and aggregation of micelles. At certain high concentration (18-20%) and temperature (higher than CGT), Poloxamer micelles get self-assembled and packed in an order that results in the transition of sol to gel state called thermo-sensitive gel [23, 24]. Moreover Pluronics are FDA approved biocompatible polymer for oral, injectable, topical, inhalation and ophthalmic preparation [24]. However one limitation associated with PF-127 hydrogels is that they are mechanically unstable, cannot maintain their structural stability and get easily dissolved in physiological environment which has limited their load-bearing applications. Researchers and scientists has made various efforts including physical or chemical modifications of PF127 to improve their mechanical performance and prevent their dissolution. For example, Hsu et al., 2009 synthesized thermoresponsive hydrogels based on hyaluronic acid and PF127 for the sustained delivery of cisplatin [25]. Similarly Guo et al., 2009, synthesized hydrogels consisting of linoleic acid cross-linked with PF127 loaded containing Paclitaxel [26], while Esther et al., 2014 prepared of poly (methyl vinyl ether-co-maleic anhydride) cross-linked with PF127 for the sustained delivery of protein respectively [10].

Poly (2-acrylamido-2-methylpropane sulfonic acid) (AMPS) $[(CH_2-CH-CONH-C-(CH_3)_2-CH_2SO_3H)_n]$ which is a strongly acidic ionic monomer behaves as polyelectrolyte and is easily dissolved in water. AMPS has great importance in hydrogel applications, however this monomer has rarely been used in hydrogel drug delivery system. AMPS received huge attention in recent years due to the

presence of strongly ionizable sulfonate groups in its chemical structure. Due to the polyelectrolyte nature of AMPS, the hydrogels prepared from AMPS show pH independent swelling behavior and exhibit complete dissociation in entire pH range [27, 28].

Herein, we prepared a series of PF127 based thermoresponsive *insitu* gelling depot hydrogels grafted with a hydrophilic monomer, 2-acrylamido-2-methylpropane sulfonic acid (AMPS) containing thixotropic properties by combination of cold and free radical polymerization technique. Current study is based on two objectives. The main objective of this study include the development of mechanically stable thermoresponsive *insitu* depot hydrogels by chemically grafting PF127 on a hydrophilic monomer AMPS and the second objective was to enhance the bioavailability of hydrophobic drug (Curcumin) *via* developing these depot hydrogels. The developed depot formulations were further evaluated for sol-gel phase transition, gelation time, rheological measurement and swelling experiments at various pH and temperature values. The thermoresponsive depot hydrogels were further used to study the *invitro* release of hydrophobic chemotherapeutic drug (Curcumin) in various dissolution media and at different temperature programs. The depot hydrogels were further subjected to the cytocompatibility and cell cytotoxicity *via* Methyl thiazolyl tetrazolium (MTT) assay against L929 cell lines, Human cervical (HeLa) and breast cancer cell lines (MCF-7). *In vivo* safety evaluation was conducted in rabbits through subcutaneous route to assess the acute toxicity. Selected samples were subjected to proton nuclear magnetic resonance spectroscopic analysis (^1H NMR), fourier transform infrared spectroscopy (FTIR), differential scanning calorimetry (DSC), thermogravimetric analysis (TGA) and scanning electron microscopy (SEM) to study the chemical structure confirmation, thermal properties and morphology of hydrogel samples.

2. Materials and methods

2.1. Materials

All materials were reagent grade and obtained from Aldrich unless otherwise noted. Pluronic F127 (PEO₉₉-PPO₆₅-PEO₉₉) (poloxamer 407) (Molecular weight, 12500 Da) was purchased from Sigma Aldrich and used without further treatment. 2-Acrylamido-2-methylpropane sulfonic acid (AMPS) (MW= 207.25 g/mol). Curcumin was purchased from Daejung chemical company, Korea with 95% purification. N, N-Methylene-bis-acrylamide (MBA) (Fluka, Switzerland). Ammonium persulphate ((NH₄)₂S₂O₈, APS), Acetic acid (Sigma Aldrich, Germany). Sodium dihydrogen phosphate, sodium hydroxide, sodium chloride were analytical reagents (Daejung, Korea). Deionized water was used throughout the experiments. Fetal bovine serum (FBS), RPMI 1640 medium, penicillin, streptomycin, Trypsin–ethylenediaminetetraacetic acid (EDTA) (0.5% trypsin, 5.3 mM EDTA tetra-sodium) and other chemicals were purchased from Invitrogen (CA, USA).

2.2. Synthesis of chemically grafted Poly (PF127-g-AMPS) thermoresponsive *insitu* gels

Chemically grafted thermosensitive Poly (PF127-g-AMPS) *insitu* gels were synthesized in various ratios of polymer, monomer, drug and cross linker as shown in Table 1 *via* a combination of cold and free radical polymerization method. The procedure is briefly described here;

For the preparation of Poly (PF127-g-AMPS) *insitu* gels, a weighed amount of thermosensitive polymer (PF127) was first dispersed in cold distilled water with continuous stirring at 400 RPM for 1 h according to the feed ratio composition given in Table 1. The polymer dispersion was then stored in refrigerator at 4°C for 24 h to obtain a clear polymeric solution. In second step, an appropriate amount of AMPS was dissolved with continuous stirring at 300 RPM for 30 minutes in distilled water at room temperature. With continuous stirring, a weighed amount of ammonium persulphate as

initiator was added to AMPS solution to create free radicals. After proper mixing, the AMPS-initiator solution was then added drop wise to the previously prepared PF127 solution and was kept at stirring for 1 h to obtain the copolymer solution. N, N-Methylenebisacrylamide (MBA) was dissolved in specific amount of distilled water and after complete dissolution was added drop wise to the already prepared copolymer solution. The final mixture was continuously stirred under nitrogen bubbling for 1 h [29, 30]. The final copolymer mixture was then sterilized by passing through filter paper and then further evaluated for their physicochemical properties i.e. T_{sol-gel}, rheology measurement, clarity of the formulations, *invitro* drug release, cytotoxic studies and characterization tools. Fig. 1 indicates the presumptive structure of Poly (PF127-g-AMPS) thermoresponsive gels.

2.3. Preparation of curcumin loaded thermoresponsive *insitu* gels

For the preparation of curcumin loaded Poly (PF127-g-AMPS) *insitu* gels, curcumin (10 mg/ml) was dissolved in specific amount of methanol and was added to copolymer solution after complete mixing with continuous stirring. The drug loaded solution was poured into pre-labeled test tubes. The copolymer solutions with the T_{sol-gel} between 32-37°C at digital water bath were selected to be used as drug delivery depot system for curcumin.

2.4. Clarity of the *insitu* gel formulations

The clarity of the *insitu* thermosensitive polymeric solutions and formed gel was observed visually at various temperature values i.e. 4°C, 25°C and 37°C [30].

2.5. Determination of phase transition temperature (T_{sol-gel})

Tube inversion method was used for the determination of the phase transition temperature (T_{sol-gel}) of all the formulations [24]. From each formulation reported in Table 1, 5 ml solution was transferred to test tube with a diameter of 1.0 cm sealed with parafilm. The test tube was placed in digital water bath (IKA, ETS-D5, Staufen, Germany) maintained at 20°C. Each time, the hydrogel sample was slowly heated at a rate of 1°C/min, from 20°C to the temperature at which the meniscus would no longer move on tilting through 90°. The gelation time of hydrogel samples was determined using the same method at 32°C. Briefly, the hydrogel solution (5 ml) was added to a test-tube (10 ml), placed in a digital water bath at 32°C and time measurements initiated. The sample flowability was observed every 10 s by tilting the tubes. The time at which flowing of the samples stopped was taken as the gelation time and the values were recorded.

2.6. Rheological determination

The viscoelastic properties of chemically grafted Poly (PF127-g-AMPS) depot formulations were measured using AR-2000 rheometer (TA instrument, USA) fitted with 40 mm diameter parallel plate with a working gap of 1000 µm. The rheological properties were monitored by conducting the time sweep measurements at 30°C at a constant oscillatory frequency of 1 rad/s. Temperature sweep experiments were conducted by changing the temperature of circulating water bath fitted with the rheometer over 25-40°C at a heating rate of 2°C per min. The dependence of viscosity, storage modulus (G') and loss modulus (G'') on temperature was evaluated at frequency of 1 rad/s and shear rate of 0.1 Pa in oscillation mode. The viscosity of the formulations as a function of the increasing shearing rate (0.1-10 s⁻¹) was also monitored in flow model.

In order to investigate the stability of the gel formulations at LCST (33°C), the gel formulations were subjected to frequency sweep test. The stability of the formulations was evaluated by observing the change in storage modulus (G') and loss modulus (G'') as a function of strain and frequency. The

frequency sweep test for stability determination was conducted in the frequency range of 0.01-50 Hz under controlled strain of 1% [24, 31].

2.7. Optical transmittance and temperature induced changes

The transmittances of chemically grafted Poly (PF127-g-AMPS) gels were measured at variable temperatures using UV-Visible spectrophotometer (UV-1601 Shimadzu Corporation, Kyoto, Japan) at 450 nm. The transmittances for all the samples were measured using 10 mm × 10 mm × 40 mm disposable cuvettes at digital water bath. The bath temperature was varied from 25°C to 45°C and every test sample was maintained at that temperature for 5 mins before analysis for transmittance [24, 32].

2.8. Swelling experiments

The swelling behavior of the chemically grafted thermoresponsive Poly (PF127-g-AMPS) *insitu* gels was analyzed in USP phosphate buffer solutions (PBS, 5mM) of different pH values (7.4 and 1.2) and at variable temperature (25°C, 37°C) in sealed containers. At regular and specific time intervals, the swollen hydrogels discs were removed from the buffer solution, wiped with a soft tissue paper before being weighed and returned back to the solution until equilibrium was maintained. The ionic strength of the buffer solution was maintained by the addition of appropriate amounts of NaCl. The swelling ratio was determined by the following equation [33];

$$\text{Swelling Ratio (Q)} = \frac{W_s - W_d}{W_d} \quad (1)$$

Where W_s and W_d indicates the weight of hydrogel samples in swollen and dry state respectively.

2.8.1. Swelling–deswelling–reswelling tests

Chemically grafted thermosensitive Poly (PF127-g-AMPS) *insitu* gels were subjected to oscillatory heating and cooling cycles in PBS of pH 7.4 at variable temperatures. For swelling ratio (SR) measurements, the hydrogel discs were immersed in solutions for 2 h at 25°C and pH 7.4, and then placed afterwards in solution for 2 h at 37°C and then again immersed in solution for 2 h at 25°C. This cycle of swelling experiment was continuously run. Each time, the weight of the swollen disc was noted prior to each immersion. The swelling ratio (SR) was calculated by the following equation [33].

$$Q = \frac{W_s - W_d}{W_d} \quad (2)$$

Where Q indicates the swelling ratio while, W_s and W_d indicates the weight of hydrogel samples in swollen and dry state respectively.

2.9. Solvent diffusion coefficient

Diffusion coefficient (D) represents the amount of substance passes through a unit area in unit time across the concentration gradient. For the determination of diffusion coefficient of the hydrogels, dried discs were soaked in basic medium (pH=7.4) due to their higher swelling. Diffusion coefficient of the hydrated gels was calculated by gradually drying the swollen gels at room temperature till

constant weight. Diffusion coefficients of the chemically grafted *insitu* depot gels were calculated by the following equation [16].

$$D = \pi \left(\frac{h \cdot \theta}{4 \cdot Q_{eq}} \right)^2 \quad (3)$$

Where D refers to the diffusion coefficient of the hydrogels, Q_{eq} refers to the equilibrium swelling degree of the *insitu* depot gel, θ is the slope of the linear part of the swelling curves, and h refers to the initial thickness of gel disc in dry state.

2.10. Networking Parameters of Poly (PF127-g-AMPS) *insitu* depot gels

2.10.1. Molecular weight between cross-links (M_c)

Molecular weight between crosslinks refers to the crosslinking degree of the developed hydrogel network. Flory-Rehner theory was used for determining the average molecular weight (M_c) values of Poly (PF127-g-AMPS) depot hydrogels. Average molecular weight (M_c) values were calculated by using the following equation [16].

$$M_c = - \frac{d_p v_s \left(v_{2,s}^{1/3} - v_{2,s}/2 \right)}{\ln(1 - v_{2,s}) + v_{2,s} + x v_{2,s}^2} \quad (4)$$

2.10.2. Volume fraction of polymers

Polymers volume fraction ($v_{2,s}$) of the swollen depot hydrogel sample refers to the amount of solvent that a hydrogel sample may entrap in its porous structure. Following equation was used for the determination of polymer volume fraction;

$$V_{2,s} = \left[1 + \frac{d_h}{d_s} \left(\frac{M_a}{M_b} - 1 \right) \right]^{-1} \quad (5)$$

Where d_h and d_s indicates the densities (g/ml) of the gel sample and solvent respectively. M_a and M_b refers to the masses (g) of the gel sample in swollen and dry state respectively. $V_{2,s}$ (ml/mol) represent the volume fraction of the hydrogel in the equilibrium state [7, 16].

2.10.3. Solvent interaction parameters (χ)

To look into the compatibility of the polymers in Poly (PF127-g-AMPS) chemically grafted depot gels with the surrounding fluids, solvent interaction parameters were measured. For the determination of solvent interaction parameter, Flory-Huggins theory was used. Following equation was used to calculate the solvent interaction parameter values.

$$\chi = \frac{\ln(1 - v_{2,s}) + v_{2,s}}{v_{2,s}^2} \quad (6)$$

Where $V_{2,s}$ (ml/mol) refers to the volume fraction of gel sample in swollen equilibrium state and χ indicates the Flory–Huggins solvent interaction parameters [7].

2.11. Determination of percent crosslinking

For the determination of percent crosslinking, chemically grafted Poly (PF127-*g*-AMPS) dried hydrogel discs were placed in distilled water (50 ml) at room temperature and stirred for 24 h to dissolve the uncrosslinked portions in the gel network. After 24 h, the hydrogel discs were taken out and thoroughly washed with distilled water and acetone to further remove the untrapped remaining portion. The swollen disc was then dried at 37°C until constant weight was obtained. Percent crosslinking was calculated by the following equation [34];

$$\text{Percent Crosslinking} = \frac{W_2}{W_1} \times 100 \quad (7)$$

Where W_1 and W_2 indicates the weights of dried discs before and after experiment. The observed values indicates the average of at least three different measurements.

2.11. Drug contents determination

The drug contents from loaded chemically grafted Poly (PF127-*g*-AMPS) gels were measured by extraction method. A 10 g of the curcumin loaded sample (100 mg/ml) was cut into discs of 7 mm in diameter. The amount of entrapped drug was calculated by extracting the loaded hydrogel samples using 25 ml of phosphate buffer solution (pH 7.4, 5 mM) as extracted solvent for 24 h. The solution was filtered to remove the polymeric debris. Drug concentration in pooled extract was determined spectrophotometrically using UV-Visible spectrophotometer (UV-1601 Shimadzu) at 421 nm [7]. All of the samples were analyzed in triplicate for drug contents determination. The drug loading percentage was calculated by using the following equation;

$$\text{Drug loading \%} = \frac{W_T - W_s}{W_T} \times 100 \quad (8)$$

Where W_T is the total drug content in the gel and W_s is the drug content in the supernatant.

2.12. Grafting efficiency determination

The quantity of the grafted PF127 to AMPS was estimated by the method reported earlier by Zhang L et al [35]. The efficiency and percentage of grafting were calculated by calculating the difference of weight before and after grafting reactions by the following equation;

$$\text{Grafting Efficiency \% (GE)} = \frac{W_g - W_{PF127}}{W_{AMPS}} \times 100 \quad (9)$$

Where W_g , W_{PF127} , W_{AMPS} are the weights of purified grafted copolymer, Pluronic (PF127) and monomer AMPS respectively.

2.13. *Invitro* curcumin release

The *invitro* curcumin release experiments for chemically grafted Poly (PF127-*g*-AMPS) gel formulations were carried out in glass vessels using USP dissolution apparatus-II (Pharmatest type PT-DT 7, Germany) in USP phosphate buffer solution (pH 7.4, 5 mM) and buffer solution of pH 1.2 (0.1 M HCl) respectively. The release experiments were carried out as a function of variable solution

temperature programs (25°C, 37°C). The drug loaded discs were placed in glass containers containing dissolution medium of desired pH and at designated temperature. At regular periods of time interval, 5 ml sample was withdrawn from the container and analyzed for release as a function of time using UV-Visible spectrophotometer (UV-1601 Shimadzu) at 421 nm. The withdrawn sample was replaced by fresh media [36]. The cumulative release of curcumin was calculated by using following equation;

$$\% \text{ Drug Release} = \frac{5C_n + 2\sum C_{n-1}}{m_0} \times 100 \quad (10)$$

Where: 5 indicates the volume of release medium, ml; C_n and C_{n-1} refers to the concentrations of drug (mg/L) in the releasing medium after n and $n-1$ withdrawing steps respectively; 2 indicates the volume of supernatant withdrawn for analysis, ml; n is the number of withdrawing steps of releasing medium; m_0 is the amount of drug (mg) loaded in gel samples.

2.14. Drug release kinetics

Drug release kinetics was assessed by fitting the *in vitro* experimental data obtained to various mathematical models [7, 24].

2.14.1. Zero order kinetic model

$$Q_t = Q_0 + K_0 t \quad (11)$$

Where Q_t is the amount of drug dissolved in time t , Q_0 is the initial amount of drug in the solution and K_0 is zero order release constant.

2.14.2. First order kinetic model

$$\ln M_t = -k_1 t + \ln M_0 \quad (12)$$

M_0 is the initial amount of curcumin and M_t is the remaining amount of curcumin at time t and k_1 is the first order rate constant.

2.14.3. Higuchi model

The model relates cumulative drug release versus square root of time as shown in Eq. (13).

$$M = k_H t^{1/2} \quad (13)$$

Where M is the amount of curcumin released at time t and k_H is the Higuchi rate equation

2.14.4. Korsmeyer–Peppas model

This model relates exponentially the drug release to the elapsed time. The equation is given as

$$\ln (M_t / M_\infty) = \ln k_p + n \ln t \quad (14)$$

(M_t/M_∞) is the fraction of drug released at time t and n is the slope which determines the type of diffusion from the polymer matrix.

2.15. *Invitro* degradation

Invitro degradation of chemically grafted Poly (PF127-*g*-AMPS) hydrogels was determined with respect to weight loss method. Each hydrogel sample was weighed initially (W_0) and placed in glass vials (10 ml) containing (5 ml) 0.1M PBS (pH= 7.4). The glass vials were incubated at 40 rpm in a shaking incubator at 37°C over an extended period, with continues PBS replenishment. At specified time intervals, hydrogels samples were quickly frozen at -80°C, then lyophilized and weighed (W_t). The weight loss ratio was determined using the following equation [37];

$$\text{Weight Loss (\%)} = \frac{W_0 - W_t}{W_0} \times 100 \quad (15)$$

Where W_0 and W_t refers to sample weights before and after degradation, respectively.

2.16. Cell lines and cell culture

2.16.1. Cell culture conditions

Human Cervical (HeLa cells), Breast (MCF-7) cancer cell lines and Vero Cell lines (African green monkey kidney cell lines) were cultured in Minimum Essential Medium (MEM) containing RPMI-1640 supplemented with l-glutamine (2 mM), penicillin (100 U mL⁻¹) and streptomycin (100 µg mL⁻¹) accompanied with 10% fetal bovine serum (FBS). The cells were stored in incubator supplied with 5% CO₂ at a constant temperature of 37°C. After attaining 90% confluency, the cells were detached from the flask with Trypsin-EDTA and the cell suspension was centrifuged at 3000 rpm for 3 minutes and then resuspended in the growth medium for further experiments.

2.16.2. Cells growth inhibition study

Methyl thiazolyl tetrazolium (MTT) assay was performed to evaluate the cell growth inhibition assessment of blank and drug loaded gel samples. The cells previously cultured were seeded in 96 well plate treated with a range of 100 µl of pure curcumin solution, copolymer solutions (containing the same drug concentrations) and blank copolymer solutions followed by incubation for 24 h at 37±1°C. The drug-loaded hydrogels were formed by incubating the plates at 37°C at which point the polymer solution transitioned from a liquid to a gel state. Cells (untreated wells) incubated in culture medium alone devoid of the formulations served as a negative control for cell viability and the wells treated with Triton X100 and free drug solution were used as positive control respectively. The absorbance's of the solution were measured on a Bio-Rad 680 microplate reader at 490 nm. Cell viability (%) was calculated according to the following equation [24,38,39]:

$$\text{Cell Viability \%} = \frac{A_{\text{sample}}}{A_{\text{control}}} \times 100 \quad (16)$$

Where A_{sample} and A_{control} are the absorbance's of the sample and control wells, respectively. The measurements were performed in triplicate.

2.17. Acute toxicity study through subcutaneous route

The acute toxicity study of developed optimized thermoresponsive injectable hydrogels was evaluated in rabbits through subcutaneous route following Food and drug administration authority (FDA, 2005) and laboratory animal science association, 2009 guidelines [40]. The study was approved by Pharmacy Research Ethics Committee (PREC), Faculty of Pharmacy and Alternative Medicine, The Islamia University of Bahawalpur (Ethics approval letter notification no: 49-2017/PREC).

2.17.1. *In vivo* biocompatibility evaluation of thermoresponsive Poly (PF127-g-AMPS) injectable hydrogels

Owing to good cytocompatibility and biodegradability of thermoresponsive *insitu* Poly (PF127-g-AMPS) gel formulation, no lethal dose (LD50) or median lethal dose has been determined. Therefore maximal tolerance dose (MTD) method has been adopted for toxicity evaluation of thermoresponsive gel formulations via subcutaneous route. Twelve rabbits (12) with average body weight of 2.50 kg were properly housed in wooden box. The rabbits were divided equally into two (2) groups; gel formulation group (n=6) and control group (n=6). All rabbits were kept in fasting state for 12 h before dosing provided excess to free water. Hydrogel group was administered 1000 mg/ml (total 4 ml, 2 g/kg) of Curcumin loaded formulation, while control group was treated with equal amount of normal saline. All rabbits were observed continuously for 14 days twice in a day. The rabbits were observed for general conditions (hair, feces, activity, behavior pattern, energy, clinical signs) mortality and body weight. At 14th day, all rabbits were sacrificed and gross histological examination was conducted to observe the major pathological changes [40].

2.17.2. Histopathological examination

After rabbit's scarification on 14th day, the samples were collected from major organs like (liver, heart, spleen, kidney and lung) and preserved properly in 10% formalin buffered and embedded subsequently in paraffin. The samples were then stained for histopathological examination with haematoxylin-eosin (H & E). For toxicity investigation of *insitu* Poly (PF127-g-AMPS) gel formulations, light microscope was used for major histopathological changes. Moreover at 14th day before scarification, a blood sample was collected from rabbits for routine blood analysis (WBC, RBC, eosinophils, basophils count, Platelets, packed cell volume, mean cell volume and hemoglobin) using hematology analyzer (SYSMEX 100, Germany) [40].

2.18. Characterization of gels

2.18.1. ¹H Nuclear magnetic resonance (NMR) spectroscopic analysis

To confirm the monomer, polymer and grafted copolymer structure, samples were subjected to ¹H-NMR (400 plus spectrometer, Bruker Ltd., UK) analysis using deuterium oxide (D₂O) (as a solvent) and tetramethylsilane (TMS) as internal standard at a frequency of 400 MHz. The chemical shifts were represented in parts per million downfield (ppm) [37].

2.18.2. Attenuated total reflectance-FTIR spectroscopic analysis

To determine the possible interactions, the samples were analyzed using ATR-Fourier transformed infrared (FTIR) spectroscopy. Briefly FT-IR spectra of pure PF127, pure AMPS, curcumin, and Poly (PF127-g-AMPS) gel samples were recorded on FTIR Accutrac FT/IR-4100 Series (Jasco, Essex, UK) at room temperature equipped with MIRacleTM software from 4000 to 600 cm⁻¹, with a resolution of 8.0 cm⁻¹ [7,36].

2.18.3. Thermogravimetric analysis (TGA)

The thermal properties of selected samples were measured by Thermogravimetric analyzer (Model: DTG 500, Shimadzu Corporation, Japan). Decomposition thermograms of TGA were recorded from 25°C to 300°C at heating rate of 10°C/min under nitrogen gas flow rate of 30 ml min⁻¹ [37].

2.18.4. Differential scanning calorimetry (DSC)

DSC 600 (Perkin Elmer, UK) thermal apparatus was used to perform the thermal analysis of the copolymer hydrogel samples under continuous nitrogen purging. Selected samples in quantity of 5 mg were sealed in an aluminum holder and heated in the temperature range of 20-300°C. The heating rate was maintained at 20°C/min. The heat flow (DSC curve) was recorded simultaneously as a function of temperature. The temperature and heat flow calibration was conducted using a pure indium standard at the same heating rate [37].

2.18.5. Scanning electron microscopy (SEM)

Structural morphologies of chemically grafted Poly (PF127-*g*-AMPS) gel samples were evaluated using scanning electron microscopy (SEM). Analyses of samples were performed using (Hitachi TM 3030 digital scanning electron microscope; JEOL Ltd, Tokyo, Japan). Dried hydrogel discs were powdered to an optimum particle size and mounted on an aluminum stub with double adhesive tape. Surface and cross section morphology of chemically grafted Poly (PF127-*g*-AMPS) gels were analyzed by taking photomicrographs. An accelerating voltage of 15 KV, chamber pressure of 0.6 mm Hg and at various magnifications having working distance of 10–25 mm, was used to scan the samples [24, 41].

2.19. Statistical analysis

Data are presented as percentage or mean $\pm$ standard deviation (SD). The difference of parameters are statistically tested for significance by one-way analysis of variance (ANOVA) using Graph Pad InStat (GraphPad Software, Inc., La Jolla, CA, USA) or Origin (v6.0; Microcal™ Software, Inc., Northampton, MA, USA) programs. Statistically significant values were defined as $P < 0.05$.

3. Results

3.1. Clarity of the *insitu* depot gels

The clarity of the *insitu* polymeric solutions and developed hydrogels reported in Table 1 were observed visually. All the polymeric solutions were observed clear at all gelation temperatures. The developed gels were also found clear and transparent at all temperatures i.e. 4°C, 25°C and 37°C indicating the solubility of all the added substances.

3.2. Sol-gel phase transition and mechanism of gelation

In order to determine the phase change of chemically grafted Poly (PF127-*g*-AMPS) gel samples, tube inversion method was used. The gelation temperature ($T_{\text{sol-gel}}$) and gelation time (T_g) are two key parameters for thermoresponsive *in situ* forming of depot hydrogels. As shown in Table 1, the gelation temperature and gelation time varies with the changing concentration of PF127 and AMPS and cross linker. It was investigated from the results in Table 1, that gelation temperature and time increases with the increase of AMPS concentration (PFAM-1 to PFAM-3) in gel network. This is

because of the fact that AMPS is hydrophilic monomer and by increasing its concentration in the feed composition, the activity of the hydrophobic groups is reduced that lead to the reduced hydrophobic interactions and alternatively lead to the increased gelation temperature and time. It was observed that by increasing the concentration of PF127 (PFAM-4 to PFAM-6), gelation temperature and time decreases as displayed in Table 1. PF127 is a series of PEO–PPO–PEO tri-block copolymers, in which PPO occupied in center form the core of Pluronic micelles while the hydrophilic PEO blocks forming the micelle shells. With the increase of polymer concentration, the hydrophobic groups (PPO blocks) which are involved in intermolecular hydrophobic interactions with the neighboring molecules increases creating a transient 3D polymer network above a critical gelation temperature. Moreover above $LCST > 32^{\circ}C$, the Pluronic micelles also increases in the gel network that in turn leads to the formation of thermoreversible hydrogels *via* intermicellar entanglements between PPO segments. Table 1 also indicates the effect of increasing curcumin contents on gelation temperature and time. It was observed that with increasing curcumin contents (PFAM-7 to PFAM-9), the gelation time and temperature decreases. This is because with increasing curcumin contents, the hydrophobicity of the gel network increases and decrease in gelation time and temperature is suggested to be due to the increased hydrophobic interactions and quicker micellar formation above the LCST of the formulation. Fig. 1 indicates the presumptive structure of Poly (PF127-*g*-AMPS) thermoresponsive gels. Fig. 2 indicates the phase transition of chemically grafted thermoresponsive gel formulations below and above CGT and schematic representation of chemical crosslinking between PF127 and AMPS.

3.3. Rheological determinations

The successful applications of injectable hydrogels are governed by several key factors including their syringibility, loading capacity of active moiety and release characteristics of the active pharmaceutical agent. Moreover the release behavior from injectable hydrogels at physiological conditions depends upon the mechanical strength of the depot formulations, which can be assessed by gaining an insight into their rheological characteristics. In order to assess the gelation at body temperature and mechanical properties of injectable hydrogels at body temperature, the rheological profile of developed formulations was observed using AR2000 rheometer.

3.3.1. Time sweep test

The buildup rate of gel samples prepared as by method described above with time was investigated by gaining an insight to their (G') and (G'') using AR2000 rheometer. The setup of the experiment was timed to 300 seconds. It is generally known that G' provides the information about the elastic nature of the material while G'' refers to the viscous nature of the material. It was observed from the time sweep test that for all the samples in the start of experiment, G'' was initially greater which refer to the sol state of the samples. However with time the setting of the gel samples occur with G' becomes equal to G'' at specific point referring to the crossover point (where $G'=G''$) of the samples. This crossover point refers to sol-gel transition of samples. The point where $G' > G''$ refers to the dominant elastic state of the samples suggesting the gel state of the samples. Fig 3A, B shows the change in (G') and (G'') of the gel samples as function of reaction time.

3.3.2. Temperature sweep test

The change in values of (G') and (G'') were evaluated over an extended temperature range as function of increasing temperature by conducting temperature ramp test. The temperature of 40 mm stainless steel plate was varied with circulating environment system for temperature control attached to AR2000 rheometer. It was found from temperature sweep experiment that PF127 which provides a thermoresponsive block did not show sol-gel conversion at 10% with AMPS within the acceptable

temperature range (32-37°C). It was observed that initially (G'') values were dominating referring to sol state of the formulations. However with temperature and contents increase, an increase in (G') values was observed indicating sol-gel conversion at specific temperature point. This is because of the fact that below CGT of the formulations, the copolymers exist as unimers or in relaxed state in solution. However with temperature increase the unimers aggregate to form micelles due to the dehydration of hydrophobic groups present in copolymer gel structure. Fig. 3C indicates the change of (G') and (G'') along with temperature for chemically grafted Poly (PF127-g-AMPS) *insitu* depot hydrogels.

3.3.3. Frequency sweep test

The mechanical stability of injectable hydrogels at body temperature are defined by change of (G') and (G'') values with frequency variation. In our experiments, the values of G' and G'' were evaluated as a function of frequency variation between 0.01-50 Hz under controlled strain of 1%. It was found that, G' was found greater than G'' indicating the stable state of the injectable hydrogel formulations at body temperature. It was concluded that chemically grafted Poly (PF127-g-AMPS) *insitu* depot hydrogels can provide prolong drug release owing to their stable structure at physiologic conditions. Fig. 3D, E indicates the change of G' and G'' as function of variable frequency for chemically grafted Poly (PF127-g-AMPS) gel formulations.

3.3.4. Continuous ramp test

The viscoelastic nature of chemically grafted Poly (PF127-g-AMPS) gel formulations was observed as function of increasing shear rate from 0-10 1/s for 10 minutes in flow mode by conducting continuous ramp test using AR rheometer 2000. It was found that all samples of Poly (PF127-g-AMPS) gel formulations at various concentrations showed a progressive decrease in viscosity with increasing shear rate at room temperature (< CGT). This behavior of gel samples with increasing forces indicates the shear thinning property probably suggested because of the dissociation of chemical crosslinks in gel network. Fig. 3F indicates the change in viscosity of gel samples at variable concentrations as a function of increasing shear rate.

3.4. Transparency assessment of formulations

The phase transition of chemically grafted Poly (PF127-g-AMPS) gel formulations at physiologic conditions was evaluated by observing the change in optical transmittances/transparent nature. Temperature dependent optical transmittance of Poly (PF127-g-AMPS) gel formulations at variable polymer concentrations was observed using UV-Visible spectrophotometer (UV-1601 Shimadzu) at 450 nm in digital water bath. For transparency measurement, the formulations were placed initially in disposable cuvettes and the temperature effect was observed by keeping the cuvettes in digital water bath. The observations were made at different temperatures below and above the CGT of the gel formulations. It was observed that initially below CGT, the copolymer formulations were colorless, transparent and homogeneous. However with temperature increase, a sharp increase in optical transparencies was observed for all formulations and become almost turbid above the LCST (32°C). These observations of the optical transmittances confirmed the CGT of the gels formulations at body temperature. Fig. 4A indicates the change in optical transmittances of formulations as a function of increasing temperature.

3.5. *In vitro* degradation study

Controlled degradation profile of biomaterial or delivery device is a key factor in the release of entrapped drug molecule or tissue engineering. The *invitro* degradation profile of chemically grafted

Poly (PF127-*g*-AMPS) gel samples containing variable polymeric contents showed that all the samples has controlled degradation rate at 37°C as shown in Fig. 4B. It was observed from degradation profile that the maximum weight loss (%) was demonstrated by gel samples with higher polymeric contents (PFAM-10) in their feed compositions owing to their increased grafting efficiency. Chemically grafted Poly (PF127-*g*-AMPS) gel samples demonstrated the surface degradation characteristics which is suggested because of random chains cutting because of hydrolysis. This type of degradation mechanism suggests long term drug delivery in a controlled fashion maintaining the mechanical properties of the hydrogels.

3.6. Percent crosslinking determinations

N, N, methylene-bis-acrylamide (MBA) was used as cross linker for chemically grafted Poly (PF127-*g*-AMPS) gel formulations. It was found that with increasing contents of AMPS, PF127 and crosslinking agent, percent crosslinking increases. This is because during reaction polymerization higher reactants concentration provides more functional groups in the reaction mixture. Due to the presence of large no of ionized functional groups, crosslinking reactions between the groups increases that lead to higher crosslinking yield and lead to dense hydrogel structure. Moreover increasing cross linker contents in the copolymer composition also result in increased percent crosslinking of the formulations. Fig. 4C indicates the effect of reaction variables on percent crosslinking of chemically grafted gels.

3.7. Grafting efficiency determination

Fig. 4D indicates the GE% with increasing monomer, polymer and cross linker contents. An increased in grafting efficiency was observed with increasing AMPS, PF127 and MBA contents in the feed composition of Poly (PF127-*g*-AMPS) gels. This increased GE is suggested because of the presence of high no of ionized functional groups that take part in the reaction. Moreover ammonium persulfate (APS) as initiator cause increased production of free ionized radicals which take part in synthesis reaction.

3.8. Swelling experiments

The response of chemically grafted Poly (PF127-*g*-AMPS) gels was observed in variable sink conditions by conducting the swelling experiments. The gel samples were cut into cylindrical circular disks (7×7 mm in dimensions) and subjected to swelling media at variable pH and temperature values. The weighed xerogel disk was placed in specific swelling media at designated temperature and the swelling ratio at various time intervals expressed as amount of solvent absorbed by the xerogel was calculated until equilibrium swelling ratio (ESR) was obtained.

3.8.1. Effect of pH on swelling behavior

The response of Poly (PF127-*g*-AMPS) gels to pH variation was investigated by observing the swelling kinetics in variable swelling medias (USP Phosphate buffer solution of pH 7.4) (PBS, 5 mM) and pH 1.2 (0.1 M HCl buffer) at constant temperature (25°C). It was observed from the swelling profile that Poly (PF127-*g*-AMPS) gels exhibited swelling behavior at both pH values, however highest swelling was observed at pH 7.4. Since AMPS is hydrophilic monomer with strong sulfonate ($-\text{SO}_3\text{H}$) ionic groups. At lower pH values, the sulfonate ($-\text{SO}_3\text{H}$) groups are in slightly ionized state causing the swelling of gels. Moreover PF127 also contain hydrophilic PEO groups facilitating the gels swelling at both pH values. However with an increased in media pH, the sulfonate ($-\text{SO}_3\text{H}$) groups strongly ionized creating the strong repulsive forces leading to enhanced swelling at higher pH values. Fig. S1A refers to the response of Poly (PF127-*g*-AMPS) gels to

swelling media of variable pH values at 25°C. Similar response to pH variation was observed by Sohail et al [42].

3.8.2. Temperature sweep swelling experiments

The swelling behavior of thermoresponsive Poly (PF127-*g*-AMPS) gels was studied as a function of two different temperature programs (25°C and 37°C) in variable swelling medias. From swelling kinetics, it was observed that gel samples exhibit highest and faster swelling at 25°C i.e. <CGT of gels in both buffer solutions. The highest swelling below CGT is suggested because of the increased intermolecular entanglements of ionized hydrophilic groups (PEO, -SO₃H) with surrounding water molecules. This phenomena leads to increased water uptake and highest swelling ratio. On the other hand at higher temperature i.e. >CGT (37°C), a decreased swelling ratio was observed at both pH values. This is because, at higher temperature, the ionized functional groups (PEO, -SO₃H) start interacting with themselves rather than associating with surrounding water molecules. This phenomena in turn leads to polymer chains contraction and repelling of water molecules and finally leads to network aggregation. Similar trends with temperature effect was observed by Clara, I et al [27]. Fig. S1B shows the swelling kinetics of Poly (PF127-*g*-AMPS) gels to temperature and pH variation.

3.8.3. Oscillatory heating and cooling cycles

Oscillatory heating and cooling cycles were performed in order to investigate the temperature dependent swelling reversibility of Poly (PF127-*g*-AMPS) gels. The oscillatory swelling cycles were performed in PBS, pH 7.4 in two fan ovens set at different temperatures. The time interval was set at 2 h between each cycle and the reversible gel response to temperature change was investigated between 25°C and 37°C. In the start of experiment, blank dried gel disks were placed in PBS 7.4 (5 mM) at 25°C and after 2 h interval, it was transferred to buffer solution at 37°C. The swelling ratios were noted accordingly. It was found that gel samples showed reversible sorption and desorption of solvent molecules at 25°C and 37°C respectively that confirms the good reversible behavior of the gels. These heating and cooling cycles were repeated several time and the data reported in Fig.S1C do not show the equilibrium swelling ratio. However this clearly indicates the temperature dependent reversible swelling behavior.

3.8.4. Effect of AMPS and MBA contents on swelling behavior

A series of samples with varying AMPS contents (PFAM-1 to PFAM-3) were prepared and subjected to swelling experiments in PBS 7.4 at 25°C and 37°C. The ratios of PF127 and MBA were kept constant in these samples in order to investigate the effect of AMPS on swelling behavior of gels. It was observed that with increasing AMPS contents in the feed composition of gels, an increased in swelling ratio was found at both temperatures. This increase in swelling ratio with increasing AMPS contents is suggested because of the presence of highest no of strong hydrophilic ionized sulfonate groups (-SO₃H). The presence of these groups in the gel structure gives enhanced swelling capacity due to increase interaction and uptake of water molecules. However temperature dominant effect was observed throughout the experiment. Fig. S2A, B refers to the effect of AMPS contents on swelling behavior of gels as a function of time and contents respectively.

MBA effect on swelling behavior was observed by developing a series of samples (PFAM-8, PFAM-10 and PFAM-11) with varying contents and subjected to swelling experiments in PBS 7.4 at 25°C and 37°C. The ratios of PF127 and AMPS were kept constant in these samples. It was observed from the swelling profile that with increasing cross linker contents in the feed composition of gels, swelling decreases accordingly. This decrease is suggested because of high crosslink density that

result in reduce pore size and compact hydrogel structure. This in turn leads to decrease water diffusion and lower swelling. This decrease swelling with increasing MBA contents were observed at both temperatures, however temperature dominant effect was investigated throughout the experiment. Fig.S2C shows the effect of variable MBA contents on swelling behavior of gels as function of time, while Fig.S2D indicates the effect of MBA on equilibrium swelling ratio of Poly (PF127-*g*-AMPS) gels at different temperature programs as function of variable contents.

3.9. Drug contents retrieved

Extraction method was adopted for determinations of encapsulated drug. Briefly all the selected samples of Poly (PF127-*g*-AMPS) gels containing 100 mg of curcumin were allowed to incubate in PBS, pH 7.4 (5 mM) at 25°C for predetermined time interval. All the samples were analyzed in triplicate for drug contents determination at 421 nm using UV-Visible spectrophotometer (UV-1601 Shimadzu, Japan). It was observed that samples with increasing AMPS contents showed higher drug loading owing to their highest swelling and more hydrophilic gel structure. It was also observed that samples with increasing MBA contents displayed decrease in drug loading. This is suggested because of higher cross linked density and compact gel network. Table 2 indicates the amount of drug retrieved from selected gel samples.

3.10. *Invitro* drug release study

Invitro drug release experiments on selected samples were conducted to investigate the response of Poly (PF127-*g*-AMPS) gels to various processing parameters i.e. pH, temperature of the dissolution media, polymeric contents and cross linker. *Invitro* release tests were conducted in various dissolution medias i.e. PBS, pH 7.4 (5 mM), pH 1.2 (0.1 M HCl).

3.10.1. Effect of pH on drug release

The pH of the dissolution medium is an important parameter that cause the release of entrapped drug from depot gel. pH sensitivity of Poly (PF127-*g*-AMPS) gels was observed in PBS, pH 7.4 (5 mM), pH 1.2 (0.1 M HCl) at 25°C. The ionization of functional groups mainly depends upon the pH of the buffer solution as well as on the pKa values of the gel components which accelerate the release of encapsulated drug. To investigate the effect of pH, curcumin loaded cylindrical circular loaded hydrogel disks (7×7 mm in dimensions) were immersed in respective buffer solution at constant temperature and the quantification of released drug was made using UV-spectrophotometer. Since hydrogels show swelling dependent release of entrapped molecules. It was observed from release profile that Poly (PF127-*g*-AMPS) gels showed maximum release in PBS 7.4. This is suggested because of strong ionization of functional groups (PEO, -SO₃H) at higher pH values which results in increased diffusion of water molecules and release of entrapped drug. This response of gels to drug release at higher pH was supported by the swelling studies of hydrogels owing to their maximum swelling in basic medium and at lower temperature.

Alternatively in acidic solution (1.2), since the functional groups (PEO, -SO₃H) in gel network remained in slightly ionized state as supported by swelling experiment, so no significant drug release was observed. However some drug release at lower pH was also observed which is suggested because of hydrophilic nature of Poly (PF127-*g*-AMPS) gels which facilitate water uptake and release of entrapped drug. Fig.5A indicates the effect of pH on curcumin release from Poly (PF127-*g*-AMPS) gels at 25°C.

3.10.2. Temperature dependent drug release

Temperature dependent release experiments of Poly (PF127-*g*-AMPS) gels were conducted at two different temperatures (25°C and 37°C) and in different dissolution mediums. It was observed from release profile displayed in Fig.5B that drug release decreases as function of increasing temperature at both pH. This is because at lower temperature (25°C) the gel network remained in hydrated state due to increased interaction of water molecules with the network functional groups. Moreover below CGT, hydrophilic nature of gels also facilitate the water uptake and drug release in turn. On the other hand, at higher temperature (37°C), gel network remained in aggregated state due to increased hydrophobic interactions of PPO groups from PF127 and alkyl groups from AMPS. Moreover the hydrophobic nature of entrapped curcumin further increase the hydrophobicity of gel network which altogether hinders the drug release. This reduction in drug release was observed at both pH values.

3.10.3. Effect of AMPS contents on drug release

Drug release study on selected samples was conducted in order to investigate the effect of variable AMPS contents. Release experiments were carried out in PBS, pH 7.4 (5 mM) and pH 1.2 (0.1 M HCl) at 25°C. An increase in drug release was observed with increasing AMPS contents in the feed composition of gels. This is because with increasing AMPS contents, the no of ionized hydrophilic sulfonate groups ($-\text{SO}_3\text{H}$) increases accordingly, which results in increased water uptake and drug diffusion in return. Alternatively in acidic solution pH 1.2 (0.1 M HCl), an increase in drug release was also observed with increasing AMPS contents, however this release was not significant due to presence of slightly ionized functional groups in gel structure and the release is suggested because of hydrophilic nature of gels. Fig. 5C-E indicates the effect of variable AMPS concentrations on drug release as a function of time and contents in variable release medias at constant temperature respectively.

3.10.4. Effect of PF127 contents on drug release

Varying PF127 contents effect on drug release was observed by developing series of samples (PFAM-4 to PFAM-6). The release experiments were conducted in PBS, pH 7.4 (5 mM) at constant temperature (25°C). It was observed that with increasing PF127 ratio in the feed composition of samples, drug release decreases. This is because PF127 contain both hydrophobic and hydrophilic groups in its network and with increasing its contents in the feed ratio, the micellar formation increases due to highest hydrophobic interaction. This in turn leads to thickening of the gel layer that retard the drug release. Fig.6A, B indicates the effect of PF127 contents on drug release as a function of time and contents respectively.

3.10.5. Effect of MBA contents on drug release

It is generally believed that changing the crosslinking density by varying cross linker contents greatly affect the release rate of encapsulated drug from gel matrix.

A series of samples (PFAM-5, PFAM-10 and PFAM-11) were prepared and subjected to release study to investigate the effect of variable MBA contents. The release experiments were conducted in PBS, pH 7.4 (5 mM) and pH 1.2 (0.1 M HCl) at 25°C. It was observed that with increasing MBA contents, drug release decreases accordingly in buffer solutions of both pH. This decrease in drug release is suggested because of growing crosslinking density and compact gel structure at higher cross linker concentrations. Fig.6C, D indicates the effect of variable MBA contents on drug release from Poly (PF127-*g*-AMPS) gels.

3.11. Networking Parameters of thermoresponsive Poly (PF127-*g*-AMPS) gels

3.11.1. Diffusion Coefficient (D)

Diffusion coefficient (D) of thermoresponsive Poly (PF127-*g*-AMPS) gels was calculated in PBS 7.4 at 25°C. It was found that D values decreases with increasing AMPS contents in the feed composition of gels. This is suggested because of higher hydrophilic nature of gels and increased swelling with increasing AMPS contents. On the other hand it was observed that with increasing MBA contents in the feed composition, D values increases accordingly. This is suggested because of compact gel structure at higher cross linker contents and lower diffusion of solvent molecules. Table S1 indicates the values of diffusion coefficient.

3.11.2. Solvent interaction parameters (χ)

To investigate the compatibility of gel components with surrounding fluids, solvent interaction parameters were calculated in PBS 7.4 at 25°C. It is generally believed that χ has inverse relationship with volume fraction of the gels that it can retain in its structure. It was assumed that grater the values of (χ), weaker will be the forces of interaction between copolymers in gels and surrounding fluids. It was observed that with increasing AMPS contents (PFAM-1 to PFAM-3), χ values decreased accordingly. This phenomena suggests the hydrophilic nature of gels and higher interaction of gel components with surrounding fluids. On the other hand with increasing MBA contents, an increased in χ values was observed. This increase is suggested because of compact gel structure and low water uptake. Table S1 indicates the χ values with increasing AMPS and MBA.

3.11.3. Polymer volume fraction

Polymer volume fraction ($V_{2,s}$) is another parameter that indicate the water uptake capacity of chemically grafted thermoresponsive Poly (PF127-*g*-AMPS) gels. It was observed from the results in Table S1 that with increasing AMPS contents, polymer volume fraction decreases accordingly. This indicate the highest water absorbing ability of gels and hydrophilic gel nature. It was also observed from the results in Table S1 that volume fraction increases with increasing MBA contents in the feed composition ratio of gels. This phenomena again refers to the compact gel structure and lower water absorbing capacity with increasing cross linker contents.

3.12. Drug release kinetics

Drug release kinetics and mechanism was studied in buffer solution of various pH values. The release kinetics and mechanism of drug release from thermoresponsive Poly (PF127-*g*-AMPS) gels was predicted by fitting the *invitro* release data obtained at 25°C to various mathematical models i.e. Zero order, First order, Higuchi, Korsmeyer–Pappas. The selecting criterion for the most suitable model was based by considering the values of regression coefficient (r) close to 1. Table 3 indicates the values of release exponent (n), release constant (k) and regression coefficient (R^2) obtained. It was observed from the results that all the samples indicated in Table 3 follow zero order with regression coefficient (R^2) values close to 1 in dissolution media of both pH values 25°C. Drug release from depot matrix and swelling of gel depot has a direct relationship. This can be explained by the fact that after depot formation absorbs the fluids from the surrounding environment which causes swelling of depot gel matrix. This in turn leads to pore openings present in the gel matrix structure. This indicates that drug release out of gel depot network occurred through pore diffusion and independent upon the concentration of drug at the application site in the depot. Additionally drug release from gel matrix also occurred through controlled degradation. Herein firstly the drug present at the gel surface released owing to surface degradation followed by bulk degradation. Release mechanism was observed by applying the Korsmeyer–Pappas model predicted from the values of release exponent “n” for all samples as shown in Table 3. It was observed from the data that all the

samples followed non-fickian diffusion with the “n” values more than 0.5 except samples (PFAM-10 and PFAM-11) which showed super case 11 transport at 7.4. This indicates that swelling and relaxation of polymer and degradation of depot are involved in drug release mechanism.

3.13. Cell growth inhibition study

The biocompatible nature of blank and efficacy of drug loaded thermoresponsive Poly (PF127-g-AMPS) gels was evaluated by MTT assay against previously cultured L929 (Mouse fibroblast cell line), Human cervical cancer cell lines (HeLa cells) and Breast cancer cell lines (MCF-7) respectively for biomedical applications. The cell cytocompatibility/cytotoxicity of gels was defined as the relative viability (%) which correlates with amount of viable cells compared with cell control.

3.13.1. *Invitro* cytocompatibility study

The compatibility of blank thermoresponsive Poly (PF127-g-AMPS) gels was evaluated against L929 (Mouse fibroblast cell line) cell lines by MTT assay. The cells previously cultured were seeded at density of 10^4 cells/well. The safe nature of developed blank formulations were evaluated by applying gels with two different contents concentrations (PFAM-6 and PFAM-11) against L929 cell lines. The untreated cells were used as negative control, while cells treated with Triton-X 100 were taken as positive control. The cells were incubated for 24 h with formulations and other negative and positive controls in 96 well plate at 37°C . Fig. 7A explain the compatible sketch of gel formulation. It is clear from the results that in comparison with negative control, blank gel samples showed good cytocompatibility (~90%) at all concentrations against L929 cell lines. However cells treated with Triton X 100 as positive control showed toxic nature and killed the cells. These results of blank gel formulations indicate the safe nature at their all concentrations and can be utilized as controlled drug delivery depot for localized and systemic drug delivery.

3.13.2. *Invitro* cytotoxicity study

The cytotoxic potential of curcumin loaded thermoresponsive Poly (PF127-g-AMPS) gels was evaluated by standard MTT assay in comparison with free curcumin solution against previously cultured (10^4 cells/well) HeLa and MCF-7 cells respectively. The untreated cells and free curcumin solution were used as negative and positive controls respectively. Previously cultured cells in 96 well plates were applied free pure curcumin solution and curcumin loaded gel formulations (100 μl) at same defined concentrations (1, 5, 10, 20, 40, 60, 80, 100 $\mu\text{g/mL}$) and then incubated for 24 h at 37°C . The cell viability was investigated by colorimetric determination using ELISA reader and the assay was terminated after 24 h incubation. It was observed from toxicity profile that free pure curcumin solution showed higher toxicity towards both cancer cell lines and drastically decreased the viability of cells after 24 h incubation at all concentrations. The toxicity potential of curcumin loaded gel formulations was also evaluated at same defined concentrations. It was concluded that curcumin loaded formulations caused controlled killing of cells as compared to free curcumin solution with highest inhibition at maximum concentrations. Fig. 7B, C indicates the cytotoxic potential of curcumin loaded thermoresponsive Poly (PF127-g-AMPS) gels.

3.13.3. IC_{50} values evaluation

The IC_{50} values were calculated for free curcumin solution and curcumin loaded thermoresponsive gels (PFAM-3 and PFAM-6) against HeLa and MCF-7 cancer cell lines from dose–response curves. For pure free curcumin solution, the IC_{50} values towards HeLa and MCF-7 cells were found to be 30 ± 0.77 $\mu\text{g/ml}$ and 27 ± 0.39 $\mu\text{g/ml}$ respectively. While for PFAM-3 against HeLa cells was found to be 19 ± 0.28 $\mu\text{g/ml}$ and 22 ± 0.54 $\mu\text{g/ml}$ against MCF-7 cells. Similarly for PFAM-6, the IC_{50} values

towards HeLa and MCF-7 cells were found to be 23 ± 0.81 $\mu\text{g/ml}$ and 21 ± 0.49 $\mu\text{g/ml}$ respectively. These lower IC_{50} values of curcumin concentration loaded in gel samples indicate the better uptake ability of cancer cells and sustained manner of gel formulations. Table 4 indicates the IC_{50} values calculated for free curcumin solution and curcumin loaded thermoresponsive Poly (PF127-*g*-AMPS) gels.

3.14. Tolerability and safety evaluation of *insitu* gels through subcutaneous route

3.14.1. General conditions monitoring

During safety profiling throughout whole toxicity study, no toxic effect was observed in any rabbit group treated with formulation controls included in study. No death occurred neither any toxic response in whole 14-days observation period. The rabbits showed normal movement, energy, eye slits, behavior, teeth, hair, eyes and oral cavity. The rabbit's response to sound, light, breathing and other stimulus was normal. No ulceration or flare was found in the skin. They displayed no nose or mouth dryness, vomit or salivation, running nose, edema or eye secretions. Rabbit's feces were found normal in color, frequency with no pus, blood and mucus. Food consumption was found equal in all treated groups with no significant difference.

3.14.2. Maximal tolerance dose (MTD)

Maximal tolerated dose refers to the dose at which the target object is observed continuously for toxicity, mortality or morbidity. So MTD indicates the highest dose tolerated by animals included for specific duration of study. Duration of dosing animals greatly affect MTD, because in some cases consecutive higher doses might not be tolerated by animals for longer duration. So animals were administered range of dosing 100-2000 mg/kg body weight. As no mortality or abnormal signs were found at higher tested dose, so MTD of Curcumin loaded in Poly (PF127-*g*-AMPS) gels was found 2000 mg/kg body weight through subcutaneous injection.

3.14.3. Histopathological examination

The samples collected from major organs of sacrificed rabbits on 14th day were observed by light microscope for any pathological changes. It was observed that after Curcumin administration through subcutaneous injection loaded in Poly (PF127-*g*-AMPS) gels, no significant pathological lesions were observed. Fig. 8 indicates the optical micrographs collected from major organs (liver, heart, spleen, kidney and lung) of rabbits groups treated with gel formulations and normal saline respectively through subcutaneous injection. No significant difference in organ weight of control and test group was detected (Group I & Group II) as shown in Table S2. Microscopic examination of tissue slides portrayed no signs of lesion, disruption, deformations, and any type of pathological changes within the vital organs of test group in comparison to control group.

3.14.4. Biochemical analysis of blood

Hematological analysis was carried out to observe effect of formulated polymeric network on biological system. In order to avoid blood coagulation, blood samples were collected in EDTA tubes immediately. Numerous parameters were perceived associated to biochemical analysis of blood as shown in Table S3. The results of biochemical blood analysis confirming biocompatibility of thermogels as findings were falling within the normal ranges. No substantial change was detected in biochemical blood analysis.

3.15. Instrumental analysis

3.15.1. NMR spectroscopic analysis

To investigate the successful grafting between AMPS and PF127 and formation of new copolymer structure, pure monomer (AMPS), polymer (PF127) and chemically grafted Poly (PF127-*g*-AMPS) gel samples were prepared in deuterium oxide (D₂O) and analyzed by NMR spectroscopy. In ¹H NMR spectroscopic analysis of PF127 indicated in Fig. 9A, the peaks appeared at 1.17 ppm and 3.61–3.77 ppm were assigned to the methyl of PPO and the methylene of PPO and PEO. In ¹H NMR spectroscopic analysis of pure AMPS shown in Fig. 9A, the signal appeared in range of 5.52–6.12 ppm are assigned to (–NH–) groups, while signals appeared at 4.70 ppm are attributed to (–CH₂SO₃H) groups. The signals appeared at 3.26 ppm corresponds to (–SO₃H) groups, 2.70 ppm (–CH–CO) groups and 1.35 ppm (–CH₃) groups respectively. In proton NMR analysis of grafted copolymer sample (PFAM) shown in Fig. 9C, new peaks were observed at 1.03 ppm (–CH₃–) groups, 1.40 ppm (–CH₂–) groups, 2.47 ppm (–CH–CO) groups, 3.39 to 3.57 ppm (proton in C₂, C₃, C₄, C₅, and C₆), 4.07 ppm (–CH₂SO₃H) groups respectively. These appearance and shift of new peaks in the ¹H NMR spectrum of grafted sample indicate the successful chemical grafting and formation of new copolymer structure.

3.15.2. FTIR spectroscopic analysis

The chemical grafting and structural confirmation of Poly (PF127-*g*-AMPS) gels was assessed through FTIR analysis. Fig. S3A shows the FTIR spectra's of pure PF127, AMPS, curcumin, blank Poly (PF127-*g*-AMPS) gel sample (PFAM) and drug loaded sample (DPFAM). In the FTIR spectra of pure AMPS, The band appeared at 1596 cm^{–1} is attributed to the C=O stretching vibration of amide group whose bending vibration appears at 1374 cm^{–1}. The band appeared at 1396 cm^{–1} is attributed to the C-N stretching and the asymmetric bending of the C-H in methyl groups. The bands appeared at 1119 and 1242 cm^{–1} are assigned to the asymmetric and symmetric stretching vibrations of the sulfonic (O=S=O) groups respectively and additionally C-S stretching observed at 752 cm^{–1}. In the FTIR spectra of pure PF127, the peak at 1692 cm^{–1} is assigned to stretching of the carboxylic acid in PF127. A peak appeared at 2981 cm^{–1} is attributed to C-H stretching. While the deeper peak at 1175 cm^{–1} show the symmetric stretching of –OH group of carboxylic acid. A peak in the range of 2000 and 2500 cm^{–1} suggested to the C-C stretching in PF127. In the FTIR spectra of gel sample (PFAM) shown in Fig. S3A, the intensity of peak in the 2000 and 2500 cm^{–1} is reduced. Moreover the peaks intensities in the range of 750 to 1800 cm^{–1} also reduced suggesting the chemical grafting reaction between AMPS and PF127. The broader peak in the range of 3110 and 3586 cm^{–1} attributed to C–H vibrations. The FTIR spectra of curcumin loaded hydrogel sample showed no additional peaks that indicate the compatibility of curcumin in copolymer network. The FTIR analysis confirmed the successful formation of Poly (PF127-*g*-AMPS) gels through chemical grafting between AMPS and PF127 by MBA as cross linker.

3.15.3. TG analysis

The thermal stability of the pure materials (PF127 and AMPS), unloaded chemically grafted thermoresponsive Poly (PF127-*g*-AMPS) gel sample (PFAM) and curcumin loaded gel sample (DPFAM) was investigated by TG analysis from 25–300°C under N₂ atmosphere. Fig. S3B shows the thermograms of pure materials and gel samples. For pure PF127, the peak degradation was observed at decomposition temperature (T_d) of 148°C. This shows a complete weight loss suggested because of the elimination of absorbed moistures. In the TG thermograms of pure AMPS, an initial peak degradation was found around 160°C followed by complete degradation associated with evaporation of the entrapped water molecules. On the other hand the TG analysis of chemically grafted gel

sample (PFAM) showed an initial weight loss at decomposition temperature (T_d) of 51°C followed by weight loss a 220°C suggesting two stage degradation. The first stage degradation was assumed to be caused by PF127 chain degradation and the second stage was found to be AMPS chain degradation. The TG thermogram of curcumin loaded gel sample (DPFAM) showed similar fashion degradation, however the sample showed complete degradation at 225°C which is suggested might be due to curcumin presence in gel network.

3.15.4. DSC analysis

The thermal behavior of thermoresponsive Poly (PF127-*g*-AMPS) gel sample (PFAM) in comparison to pure materials (PF127 and AMPS) was investigated by subjecting samples to DSC analysis. The DSC analysis were carried out under N_2 atmosphere by heating in the temperature range of 22°C to 300°C. The DSC curve of pure PF127 showed a broad endothermic peak 46°C that corresponds to enthalpy of pluronic micelles and its melting temperature (T_m). The DSC curve of pure AMPS showed sharp endothermic peak at 201°C. The DSC curve of pure curcumin exhibit n thermal signals which indicate their wide thermal stability. In the DSC curve of chemically grafted hydrogel sample (PFAM), a smaller endothermic peak was observed in the range of 53°C to 100°C indicating the presence of PF127. While some small endothermic peaks were also observed in the range of 250°C to 290°C which indicate the presence of AMPS in gel network. Moreover the DSC curve of curcumin loaded sample (DPFAM) did not show any significant changes which in turn indicate the compatibility of curcumin in gel network. This change in the melting temperatures (T_m) in DSC curve of gel sample indicate the formation of graft copolymer between PF127 and AMPS. Fig. S3C indicates the DSC spectra's of pure materials and gel sample.

3.15.5. SEM analysis

Fig. 10 indicates the surface and cross-sectional morphologies of chemically grafted thermoresponsive Poly (PF127-*g*-AMPS) gel sample. The surface morphology of gel sample showed a combined nature of smooth and porous structure. These pores are hypothized in the diffusion of water and drug molecules. The cross sectional morphological analysis at various resolutions of hydrogel samples revealed a highly inter connected porous morphology. These pores are assumed to act as channels for absorption of physiological solutions, water swellibilty and diffusion of solute (curcumin) in and out of the gel network.

4. Conclusion

Current study was based on development of novel thermoresponsive PF127 based injectable depot gel formulations and enhancement of bioavailability of hydrophobic chemotherapeutic agent (curcumin). In current study, new series of thermoresponsive *insitu* cross linkable depot formulations were synthesized based on Pluronic-127 with 2-acrylamido-2-methylpropane sulfonic acid in aqueous solution using MBA as cross linker. Rheological analysis and tube titling experiments confirmed the viscoelastic properties, mechanical strength and tunable gelation temperature of formulations above 32°C. Optical transmittance experiments confirmed the presence of thermoresponsive PF127 by rendering the gel formulations opaque with increasing temperature effect. It was concluded from the results that newly developed gel formulations exhibit maximum swelling and drug release at pH 7.4 and 25°C attributed to the presence of ionized sulfonate groups and relaxed gel state below CGT. Release kinetic suggested that pore diffusion and degradation are involved in the release of encapsulated drug following zero order kinetics. *Invitro* degradation profile indicated that gel with variable composition exhibit controlled degradation. *Invitro* cytocompatibility assay showed the biocompatible nature of gels tested against L929 cells. While *invitro* cytotoxicity study confirmed that curcumin loaded formulations has the ability to induce cell death against HeLa

and MCF-7 cancer cells. Acute toxicity study showed that blank gels are biocompatible with the body tissues administered through subcutaneous route. NMR and FTIR analysis confirmed the successful grafting of AMPS onto PF127 backbone. DSC and TGA analysis showed that developed formulations has phase transition at body temperature and are thermally stable. SEM analysis confirmed that the developed formulations has porous structure which assist in water and drug uptake and also facilitate the release of drug from gel network. Results concluded that altogether newly developed formulations has thermosensitivity and *insitu* crosslinkability and can be used as injectable drug delivery depot owing to their safe nature.

5. Disclosure statement

No potential conflict of interest was reported by the authors.

6. Acknowledgments

Authors are highly thankful to Islamia University of Bahawalpur, Pakistan for financing this study for the project under the Financial Support Research Program.

7. References

- [1] T. ALAIKOV, S.M. KONSTANTINOV, T. TZANOVA, K. DINEV, M. TOPASHKA-ANCHEVA, M.R. BERGER, Antineoplastic and Anticlastogenic Properties of Curcumin, *Ann. N. Y. Acad. Sci.* 1095 (2007) 355–370. doi:10.1196/annals.1397.039.
- [2] R. Bruck, M. Ashkenazi, S. Weiss, I. Goldiner, H. Shapiro, H. Aeed, O. Genina, Z. Helpner, M. Pines, Prevention of liver cirrhosis in rats by curcumin, *Liver Int.* 27 (2007) 373–383. doi:10.1111/j.1478-3231.2007.01453.x.
- [3] P. Anand, A.B. Kunnumakkara, R.A. Newman, B.B. Aggarwal, Bioavailability of Curcumin: Problems and Promises, *Mol. Pharm.* 4 (2007) 807–818. doi:10.1021/mp700113r.
- [4] N. Sowasod, K. Nakagawa, W. Tanthapanichakoon, T. Charinpanitkul, Development of encapsulation technique for curcumin loaded O/W emulsion using chitosan based cryotropic gelation, *Mater. Sci. Eng. C.* 32 (2012) 790–798. doi:10.1016/J.MSEC.2012.01.027.
- [5] M. Sun, X. Su, B. Ding, X. He, X. Liu, A. Yu, H. Lou, G. Zhai, Advances in nanotechnology-based delivery systems for curcumin, *Nanomedicine.* 7 (2012) 1085–1100. doi:10.2217/nmm.12.80.
- [6] A. Anitha, S. Maya, N. Deepa, K.P. Chennazhi, S.V. Nair, H. Tamura, R. Jayakumar, Efficient water soluble O-carboxymethyl chitosan nanocarrier for the delivery of curcumin to cancer cells, *Carbohydr. Polym.* 83 (2011) 452–461. doi:10.1016/J.CARBPOL.2010.08.008.
- [7] S. Khan, N.M. Ranjha, Effect of degree of cross-linking on swelling and on drug release of low viscous chitosan/poly(vinyl alcohol) hydrogels, *Polym. Bull.* 71 (2014) 2133–2158. doi:10.1007/s00289-014-1178-2.
- [8] R. V. Kulkarni, B. Sa, Polyacrylamide-Grafted-Alginate-Based pH-Sensitive Hydrogel Beads for Delivery of Ketoprofen to the Intestine: in Vitro and in Vivo Evaluation, *J. Biomater. Sci. Polym. Ed.* 20 (2009) 235–251. doi:10.1163/156856209X404514.
- [9] L. Yu, Z. Zhang, H. Zhang, J. Ding, Biodegradability and Biocompatibility of Thermoreversible Hydrogels Formed from Mixing a Sol and a Precipitate of Block Copolymers in Water, *Biomacromolecules.* 11 (2010) 2169–2178. doi:10.1021/bm100549q.

- [10] E. Moreno, J. Schwartz, E. Larrañeta, P.A. Nguewa, C. Sanmartín, M. Agüeros, J.M. Irache, S. Espuelas, Thermosensitive hydrogels of poly(methyl vinyl ether-co-maleic anhydride) – Pluronic® F127 copolymers for controlled protein release, *Int. J. Pharm.* 459 (2014) 1–9. doi:10.1016/J.IJPHARM.2013.11.030.
- [11] T. Miao, S.L. Fenn, P.N. Charron, R.A. Oldinski, Self-Healing and Thermoresponsive Dual-Cross-Linked Alginate Hydrogels Based on Supramolecular Inclusion Complexes, *Biomacromolecules*. 16 (2015) 3740–3750. doi:10.1021/acs.biomac.5b00940.
- [12] Y. Li, Y. Tan, K. Xu, C. Lu, X. Liang, P. Wang, In situ crosslinkable hydrogels formed from modified starch and O-carboxymethyl chitosan, *RSC Adv.* 5 (2015) 30303–30309. doi:10.1039/C4RA14984J.
- [13] T.-W. Chung, S.-Y. Lin, D.-Z. Liu, Y.-C. Tyan, J.-S. Yang, Sustained release of 5-FU from Poloxamer gels interpenetrated by crosslinking chitosan network, *Int. J. Pharm.* 382 (2009) 39–44. doi:10.1016/J.IJPHARM.2009.07.035.
- [14] S.-Y. Lee, G. Tae, Formulation and in vitro characterization of an in situ gelable, photo-polymerizable Pluronic hydrogel suitable for injection, *J. Control. Release*. 119 (2007) 313–319. doi:10.1016/J.JCONREL.2007.03.007.
- [15] M. Constantin, S. Bucatariu, V. Harabagiu, I. Popescu, P. Ascenzi, G. Fundueanu, Poly(N-isopropylacrylamide-co-methacrylic acid) pH/thermo-responsive porous hydrogels as self-regulated drug delivery system, *Eur. J. Pharm. Sci.* 62 (2014) 86–95. doi:10.1016/J.EJPS.2014.05.005.
- [16] N. Sivagangi Reddy, K.S. V. Krishna Rao, S. Eswaramma, K. Madhusudana Rao, Development of temperature-responsive semi-IPN hydrogels from PVA-PNVC-PAm for controlled release of anti-cancer agent, *Soft Mater.* 14 (2016) 96–106. doi:10.1080/1539445X.2016.1150294.
- [17] T. Baskan, D.C. Tuncaboylu, O. Okay, Tough interpenetrating Pluronic F127/polyacrylic acid hydrogels, *Polymer (Guildf)*. 54 (2013) 2979–2987. doi:10.1016/J.POLYMER.2013.03.066.
- [18] J.-Y. Fang, S.-H. Hsu, Y.-L. Leu, J.-W. Hu, Delivery of Cisplatin from Pluronic Co-polymer Systems: Liposome Inclusion and Alginate Coupling, *J. Biomater. Sci. Polym. Ed.* 20 (2009) 1031–1047. doi:10.1163/156856209X444493.
- [19] L. Qiu, Novel degradable polyphosphazene hydrogel beads for drug controlled release, *J. Appl. Polym. Sci.* 87 (2003) 986–992. doi:10.1002/app.11420.
- [20] G.D. Kang, S.H. Cheon, G. Khang, S.-C. Song, Thermosensitive poly(organophosphazene) hydrogels for a controlled drug delivery, *Eur. J. Pharm. Biopharm.* 63 (2006) 340–346. doi:10.1016/J.EJPB.2006.01.001.
- [21] Y.J. Kim, S. Choi, J.J. Koh, M. Lee, K.S. Ko, S.W. Kim, Controlled Release of Insulin from Injectable Biodegradable Triblock Copolymer, *Pharm. Res.* 18 (2001) 548–550. doi:10.1023/A:1011074915438.
- [22] H.J. Chung, Y. Lee, T.G. Park, Thermo-sensitive and biodegradable hydrogels based on stereocomplexed Pluronic multi-block copolymers for controlled protein delivery, *J. Control. Release*. 127 (2008) 22–30. doi:10.1016/J.JCONREL.2007.12.008.
- [23] N.A.K. Mezmarich, B.J. Love, The Kinetics of Gel Formation for PEO–PPO–PEO Triblock Copolymer Solutions and the Effects of Added Methylparaben, *Macromolecules*. 44 (2011) 3548–3555. doi:10.1021/ma200302s.
- [24] S. Khan, M.U. Minhas, M. Ahmad, M. Sohail, Self-assembled supramolecular

thermoreversible β -cyclodextrin/ethylene glycol injectable hydrogels with difunctional Pluronic[®] 127 as controlled delivery depot of curcumin. Development, characterization and in vitro evaluation, *J. Biomater. Sci. Polym. Ed.* 29 (2018). doi:10.1080/09205063.2017.1396707.

- [25] S.-H. Hsu, Y.-L. Leu, J.-W. Hu, J.-Y. Fang, Physicochemical Characterization and Drug Release of Thermosensitive Hydrogels Composed of a Hyaluronic Acid/Pluronic F127 Graft, *Chem. Pharm. Bull. (Tokyo)*. 57 (2009) 453–458. doi:10.1248/cpb.57.453.
- [26] D.-D. Guo, C.-X. Xu, J.-S. Quan, C.-K. Song, H. Jin, D.-D. Kim, Y.-J. Choi, M.-H. Cho, C.-S. Cho, Synergistic anti-tumor activity of paclitaxel-incorporated conjugated linoleic acid-coupled poloxamer thermosensitive hydrogel in vitro and in vivo, *Biomaterials*. 30 (2009) 4777–4785. doi:10.1016/J.BIOMATERIALS.2009.05.051.
- [27] I. Clara, R. Lavanya, N. Natchimuthu, pH and temperature responsive hydrogels of poly(2-acrylamido-2-methyl-1-propanesulfonic acid-co-methacrylic acid): Synthesis and swelling characteristics, *J. Macromol. Sci. Part A*. 53 (2016) 492–499. doi:10.1080/10601325.2016.1189282.
- [28] E. Karada, S. Kundakci, Ö.B. Üzümlü, Investigation of Swelling/Sorption Characteristics of Highly Swollen AAm/AMPS Hydrogels and Semi IPNs with PEG as Biopotential Sorbent, *J. Encapsulation Adsorpt. Sci.* 1 (2011) 7–22. doi:10.4236/jeas.2011.11002.
- [29] S. Yu, X. Zhang, G. Tan, L. Tian, D. Liu, Y. Liu, X. Yang, W. Pan, A novel pH-induced thermosensitive hydrogel composed of carboxymethyl chitosan and poloxamer cross-linked by glutaraldehyde for ophthalmic drug delivery, *Carbohydr. Polym.* 155 (2017) 208–217. doi:10.1016/J.CARBPOL.2016.08.073.
- [30] F. Nasir, Z. Iqbal, J.A. Khan, A. Khan, F. Khuda, L. Ahmad, A. Khan, A. Khan, A. Dayoo, Roohullah, Development and evaluation of diclofenac sodium thermoreversible subcutaneous drug delivery system, *Int. J. Pharm.* 439 (2012) 120–126. doi:10.1016/J.IJPHARM.2012.10.009.
- [31] H.R. Moreira, F. Munarin, R. Gentilini, L. Visai, P.L. Granja, M.C. Tanzi, P. Petrini, Injectable pectin hydrogels produced by internal gelation: pH dependence of gelling and rheological properties, *Carbohydr. Polym.* 103 (2014) 339–347. doi:10.1016/J.CARBPOL.2013.12.057.
- [32] B. Wang, X. Wu, J. Li, X. Hao, J. Lin, D. Cheng, Y. Lu, B. Wang, X. Wu, J. Li, X. Hao, J. Lin, D. Cheng, Y. Lu, Thermosensitive Behavior and Antibacterial Activity of Cotton Fabric Modified with a Chitosan-poly(N-isopropylacrylamide) Interpenetrating Polymer Network Hydrogel, *Polymers (Basel)*. 8 (2016) 110. doi:10.3390/polym8040110.
- [33] W.. Wan Ngah, C.. Endud, R. Mayanar, Removal of copper(II) ions from aqueous solution onto chitosan and cross-linked chitosan beads, *React. Funct. Polym.* 50 (2002) 181–190. doi:10.1016/S1381-5148(01)00113-4.
- [34] R. Jankaew, N. Rodkate, S. Lamlertthon, B. Rutnakornpituk, U. Wichai, G. Ross, M. Rutnakornpituk, “Smart” carboxymethylchitosan hydrogels crosslinked with poly(N-isopropylacrylamide) and poly(acrylic acid) for controlled drug release, *Polym. Test.* 42 (2015) 26–36. doi:10.1016/J.POLYMERTESTING.2014.12.010.
- [35] L. Zhang, L. Wang, B. Guo, P.X. Ma, Cytocompatible injectable carboxymethyl chitosan/N-isopropylacrylamide hydrogels for localized drug delivery, *Carbohydr. Polym.* 103 (2014) 110–118. doi:10.1016/J.CARBPOL.2013.12.017.
- [36] W. Wang, E. Wat, P.C.L. Hui, B. Chan, F.S.F. Ng, C.-W. Kan, X. Wang, H. Hu, E.C.W.

Wong, C.B.S. Lau, P.-C. Leung, Dual-functional transdermal drug delivery system with controllable drug loading based on thermosensitive poloxamer hydrogel for atopic dermatitis treatment, *Sci. Rep.* 6 (2016) 24112. doi:10.1038/srep24112.

- [37] S. Pentlavalli, P. Chambers, B.N. Sathy, M. O'Doherty, M. Chalanqui, D.J. Kelly, T. Haut-Donahue, H.O. McCarthy, N.J. Dunne, Simple Radical Polymerization of Poly(Alginate-Graft- *N*-Isopropylacrylamide) Injectable Thermoresponsive Hydrogel with the Potential for Localized and Sustained Delivery of Stem Cells and Bioactive Molecules, *Macromol. Biosci.* 17 (2017) 1700118. doi:10.1002/mabi.201700118.
- [38] T.M. Tamer, A.M. Omer, M.A. Hassan, M.E. Hassan, M.M. Sabet, M.S.M. Eldin, Development of thermo-sensitive poly *N*-isopropyl acrylamide grafted chitosan derivatives., (2015). <http://imsear.hellis.org/handle/123456789/166801> (accessed July 1, 2016).
- [39] H.S. Shah, R. Al-Oweini, A. Haider, U. Kortz, J. Iqbal, Cytotoxicity and enzyme inhibition studies of polyoxometalates and their chitosan nanoassemblies, *Toxicol. Reports.* 1 (2014) 341–352. doi:10.1016/J.TOXREP.2014.06.001.
- [40] Barkat K, Ahmad M, Minhas MU, Khalid I (2017) Oxaliplatin-loaded crosslinked polymeric network of chondroitin sulfate-co-poly(methacrylic acid) for colorectal cancer: Its toxicological evaluation. *J Appl Polym Sci.* <https://doi.org/10.1002/app.45312>
- [40] A. Jalil, S. Khan, F. Naeem, M.S. Haider, S. Sarwar, A. Riaz, N.M. Ranjha, The structural, morphological and thermal properties of grafted pH-sensitive interpenetrating highly porous polymeric composites of sodium alginate/acrylic acid copolymers for controlled delivery of diclofenac potassium, *Des. Monomers Polym.* 20 (2017) 308–324. doi:10.1080/15685551.2016.1259834.
- [41] M. Sohail, M. Ahmad, M.U. Minhas, L. Ali, I. Khalid, H. Rashid, Controlled delivery of valsartan by cross-linked polymeric matrices: Synthesis, in vitro and in vivo evaluation, *Int. J. Pharm.* 487 (2015) 110–119. doi:10.1016/J.IJPHARM.2015.04.013.

Table 1 Formulation sheet of *insitu* formed chemically grafted Poly (PF127-graft-AMPS) thermoresponsive injectable hydrogels

Sample Codes	PF-127 (g)	AMPS (g)	MBA (g)	APS (g)	T _{sol-gel} (°C)	Gelation Time (T _g) (Minutes)	Curcumin (mg)	H ₂ O (g)
PF-1	1	0	0	0	>37	No gelation	0	QS to 10 g
PF-2	1.5	0	0	0	36 ± 0.30	~6	0	QS to 10 g
PFAM-1	1.50	0.25	0.125	0.150	35 ± 0.70	~4	100	QS to 10 g
PFAM-2	1.50	0.50	0.125	0.150	36 ± 0.60	~4.5	100	QS to 10 g
PFAM-3	1.50	1	0.125	0.150	36 ± 0.10	~5	100	QS to 10 g
PFAM-4	1.75	1	0.125	0.150	35 ± 0.10	~4	100	QS to 10 g
PFAM-5	2.00	1	0.125	0.150	34 ± 0.20	~3	100	QS to 10 g
PFAM-6	2.25	1	0.125	0.150	32 ± 0.20	~3	100	QS to 10 g
PFAM-7	2.00	1	0.125	0.150	33 ± 0.90	~3.5	115	QS to 10 g
PFAM-8	2.00	1	0.125	0.150	33 ± 0.40	~3	130	QS to 10 g
PFAM-9	2.00	1	0.125	0.150	32 ± 0.80	~3	145	QS to 10 g
PFAM-10	2.00	1	0.150	0.150	33 ± 0.20	~3	100	QS to 10 g
PFAM-11	2.00	1	0.200	0.150	32 ± 0.70	~3	100	QS to 10 g

Table 2 Clarity of the formulations and drug contents retrieved

Formulation Codes	Clarity of Formulations	Drug Contents %
PFAM-1	+++++	87 ± 0.72
PFAM-2	+++++	91 ± 0.30
PFAM-3	+++++	97 ± 0.73
PFAM-4	+++++	91 ± 0.72
PFAM-5	++++	86 ± 0.39
PFAM-6	++++	81 ± 0.65
PFAM-7	+++++	98 ± 0.57
PFAM-8	++++	97 ± 0.29
PFAM-9	++++	91 ± 0.56
PFAM-10	+++++	82 ± 0.75
PFAM-11	++++	74 ± 0.61

Clarity: Good; +++++, Fair; ++++

Table 3 Drug release kinetics

Sample Codes	pH	Zero order kinetics		First order kinetics		Higuchi Model	Korsmeyer–Peppas Model	
		K_0 (h ⁻¹)	R^2	K_1 (h ⁻¹)	R^2	K_2 (h ⁻¹) R^2	n	R^2
PFAM-1	1.2	1.347	0.994	0.015	0.996	5.461 0.983	0.588	0.994
	7.4	3.604	0.990	0.051	0.988	14.55 0.974	0.673	0.977
PFAM-2	1.2	1.296	0.995	0.041	0.994	5.188 0.961	0.461	0.969
	7.4	4.442	0.995	0.068	0.972	17.68 0.950	0.625	0.978
PFAM-3	1.2	1.681	0.997	0.019	0.997	6.757 0.971	0.541	0.982
	7.4	5.094	0.997	0.084	0.983	20.45 0.970	0.644	0.990
PFAM-5	1.2	1.465	0.994	0.016	0.996	5.922 0.979	0.513	0.987
	7.4	3.740	0.994	0.050	0.980	14.92 0.954	0.814	0.993
PFAM-10	1.2	1.196	0.993	0.013	0.994	4.822 0.973	0.573	0.981
	7.4	3.615	0.995	0.046	0.979	12.26 0.933	1.089	0.998
PFAM-11	1.2	1.240	0.997	0.013	0.996	4.955 0.958	0.498	0.969
	7.4	3.270	0.984	0.040	0.971	12.76 0.903	1.032	0.968

Table 4 The IC₅₀ values and % inhibition of pure Curcumin solution and Curcumin loaded in Poly (PF127-g-AMPS) gels against HeLa and Breast (MCF-7) Cancer cell lines and L929 cell lines.

Sample Codes	^a IC ₅₀ (μg/ml) against HeLa Cells	^a IC ₅₀ (μg/ml) against MCF-7 Cells [%] S on Vero Cells _±	% Cell inhibition in L929 cell lines
Triton X100	-	-	82 ± 0.65
Curcumin	30±0.77	27±0.39	-
PFAM-3	19±0.28	22±0.54	8 ± 0.63
PFAM-6	23±0.81	21±0.49	7 ± 0.82

^a IC₅₀, Concentration of curcumin (or equivalent) (μg/ml) required to inhibit the cellular growth by 50% after 24 h of drug exposure, as determined by the MTT assay. Data reported are the percentages or mean ± standard deviation (n=3).

Figure 2. Sol-gel phase transition below and above critical gelation temperature and schematic representation of chemical crosslinking between PF127 and AMPS.

Figure 3. Oscillatory time sweep tests of chemically grafted gel formulations at 30°C over a period of 300 seconds. The experimental conditions were set as (Strain= 1% and Frequency= 1Hz). All the experiments were conducted ($n=3$) in triplicates (A, B). Change of (G') and (G'') over an extended temperature range (25°C to 40°C) as function of increasing temperature for thermoresponsive gel samples (C) Change in (G'') and (G') as function of increasing frequency under controlled strain of 1% (D, E) Viscosity as function of increasing shear rate ($0.1\text{-}10\text{ s}^{-1}$) for 10 minutes at 25°C (F)

Figure 4. Temperature dependent optical transmittance of chemically grafted Poly (PF127-g-AMPS) gel formulations (A) *In vitro* degradation profile of Poly (PF127-g-AMPS) gel formulations at 37°C (B) Effect of processing parameters on % grafting efficiency (C) Effect of processing parameters on percent crosslinking of Poly (PF127-g-AMPS) gel formulations (D). All experiments were conducted in triplicates (n=3).

Figure 5. Response of Poly (PF127-g-AMPS) gels to cumulative curcumin release in buffer solutions of variable pH values at 25°C (A) Effect of pH and temperature on cumulative curcumin release from Poly (PF127-g-AMPS) gels (B) Cumulative curcumin release with time from Poly (PF127-g-AMPS) gels as function of increasing AMPS contents in PBS 7.4 at 25°C (C) at pH 1.2 and 25°C (D) Cumulative curcumin release from Poly (PF127-g-AMPS) gels as function of increasing AMPS contents (E). The data indicates the mean $\pm$ SD of (n=3) individual experiments.

Figure 6. Cumulative curcumin release with time from Poly (PF127-g-AMPS) gels as function of increasing PF127 contents in PBS 7.4 at 25°C (A) Cumulative curcumin release from Poly (PF127-g-AMPS) gels as function of increasing PF127 contents (B) Effect of variable MBA contents in feed composition ratio of chemically grafted Poly (PF127-g-AMPS) gels on cumulative curcumin release as function of time in PBS 7.4 at 25°C (C) Cumulative curcumin release from Poly (PF127-g-AMPS) gels as function of increasing MBA contents (D). The data indicates the mean $\pm$ SD of (n=3) individual experiments.

Figure 8. Histopathological microscopic examinations of different rabbit tissues of control (group 1) and test (group 11)

Figure 9. ^1H -NMR spectrum analysis of (A) Pure PF127 (B) Pure AMPS (C) Poly (PF127-g-AMPS) gel sample (PFAM-6)

Figure 10. SEM analysis of chemically grafted thermoresponsive Poly (PF127-*g*-AMPS) gel sample. Surface morphology $\times 100$ magnification (A) Surface morphology $\times 250$ magnification (B) Cross-sectional morphology $\times 400$ magnification (C) Cross-sectional morphology $\times 500$ magnification (D) Cross-sectional morphology $\times 1000$ magnification.