DEVELOPMENT AND EVALUATION OF THERMOSENSITIVE IN-SITU GEL USING N-ISOPROPYLACRYLAMIDE GRAFTED TAMARIND SEED POLYSACCHARIDE FOR THE TREATMENT OF GLAUCOMA

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By

SONALI MANDAL

M. PHARM.

CLASS ROLL NO.: 002211402004

EXAMINATION ROLL NO.: M4PHP24013

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UNDER THE GUIDANCE OF

Dr. TAPAN KUMAR GIRI

Division of Pharmaceutics

Department of Pharmaceutical Technology

Faculty Of Engineering and Technology
Jadavpur University
Kolkata-700032

2024

DEPARTMENT OF PHARMACEUTICAL TECHNOLOGY

FACULTY OF ENGINEERING AND TECHNOLOGY

JADAVPUR UNIVERSITY

KOLKATA-700032

CERTIFICATE

This is to certify that SONALI MANDAL (Class Roll No.: 002211402004 and Reg. No.: 163648 of 2022-2023), has carried out the research work on the subject entitled "DEVELOPMENT AND EVALUATION OF THERMOSENSITIVE OPTHALMIC IN SITU GEL USING N-ISOPROPYLACRYLAMIDE GRFTED TAMRIND SEED POLYSACCHRIDE FOR THE TREATMENT OF GLAUCOMA" under my supervision in the Pharmaceutics Research Laboratory in the Department of Pharmaceutical Technology of this university. She has incorporated her findings into this thesis of the same title, being submitted by her, in partial fulfilment of the requirements for the degree of **Master of Pharmacy (Pharmaceutics)** of Jadavpur University. She has carried out this research work independently and with proper care and attention to my entire satisfaction.

Tapan Kumar Giri 27/08/24

Dr. Tapan Kumar Giri

Associate Professor

Department of Pharmaceutical Technology Jadavpur University

Kolkata-700 032

Dr. Tapan Kumar Giri
Associate Professor
Dept. of Pharm. Tech.
Jadavpur University
Kolkata-700 032, India

27/8/24.

Prof. Amalesh Samanta

Head of the Department

Department of Pharmaceutical Technology

Jadavpur University

Kolkata-700032

Prof. Amalesh Samanta, Ph.D.
Head
Dept. of Pharmaceutical Technology
Jadavpur University, Kolkata, India

Dipak Laha 28.8.24

Prof. Dipak Laha

Dean

Faculty of Engineering and Technology

Jadavpur University

Kolkata- 70003

DEAN
Faculty of Engineering & Technology
JADAVPUR UNIVERSITY
KOLKATA-700 032

DECLARATION OF ORIGINALITY AND COMPLIANCE OF ACADEMIC ETHICS

I hereby declare that this thesis contains literature survey and original research work by the undersigned candidate, as part of her Master of Pharmaceutical Technology studies. All information in this document have been obtained and presented in accordance with academic rules and ethical conduct. I also declare that as required by these rules and conduct, I have fully cited and referenced all materials and results that are not original to this work.

Name: SONALI MANDAL

Class Roll No.: 002211402004
Registration No.: 163648 of 2022-2023

Thesis title: DEVELOPMENT AND EVALUATION OF THERMOSENSITIVE OPHTHALMIC IN-SITU GEL USING N-ISOPROPYLACRYLAMIDE GRAFTED TAMARIND SEED POLYSACCHARIDE FOR THE TREATMENT OF GLAUCOMA

Sonali Mandal 27/08/2024

Signature with date

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Place: Department of Pharmaceutical Technology, Jadavpur University

Date: 27/08/2024

Name.: SONALI MANDAL

Class Roll No.: 002211402004

Registration No.: 163648 of 2022-2023

Department of Pharmaceutical Technology, Jadavpur University

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

Glaucoma is a serious eye disorder characterized by an increase in intraocular pressure, which leads gradually to loss of vision due to damage of the optic nerve with no symptoms. Glaucoma development may be observed due to imbalance between aqueous humor secretion and drainage processes within the ocular chamber [1]. Dorzolamide hydrochloride is a carbonic anhydrase inhibitor used in the treatment of glaucoma. Carbonic anhydrase inhibitors reduce IOP by decreasing aqueous humor secretion through the inhibition of carbonic anhydrase isoenzyme II in the ciliary processes[2]. Carbonic anhydrase inhibitors (CAIs) are available as aqueous eye drops solutions (dorzolamide), suspensions (brinzolamide) for topical application and as tablets for systemic drug delivery (acetazolamide and methazolamide) [3]. Dorzolamide hydrochloride should be applied topically to the affected eye every three hours, or hourly in case of severe infection. The recommended dosage for this is 1-2 drops of a 2% w/v solution. The ocular distribution of medicine has proven to be one of the most difficult challenges for a pharmaceutical scientist due to the particular structure of the eye, which prevents the entry of drug molecules into the desired spot. More than 90% of ophthalmic medicines available on the market are eye drops [4]. However, due to the high tear fluid turnover and quick precorneal clearance of the medicine, eye drops frequently have low bioavailability and therapeutic response [5]. The administration of eye drops frequently is correlated with patient noncompliance. If the medicine solution drained from the eye is constantly absorbed from the nasolacrimal duct, adding extra medication to the formulation to address bioavailability issues could be harmful [6]. Short precorneal residence time, poor corneal penetration , and low ocular bioavailability are the main drawbacks of this type of dosage form [7]. Many ophthalmic vehicles, such viscous solutions, ointments, gels, or polymeric inserts, have been developed to lengthen the ocular residence time of topical eye treatments in an effort to solve these issues. These vehicles have improved the corneal contact time to varied degrees. But these formulations have not been well received because of obscured eyesight (for example, ointments) or low patient compliance (for example, inserts). Therefore, achieving adequate ocular bioavailability of a medicine after topical administration is still difficult and requires attention. The creation of an in-situ gelling delivery system, which is supplied as a liquid and transforms into a semisolid gel upon exposure to the physiological environment, can solve these issues. [8]. The Latin word in-situ, which means "in its original place or in position," is the source of the phrase. The polymeric in situ gel has received more attention due to its benefits over traditional ophthalmic formulations, including simplicity of administration, decreased frequency of administration, and greater patient compliance. In situ gels can be divided into ion-activated, thermosensitive and pH-sensitive types [9] The thermosensitive in situ gel, which is one of three varieties of in situ gels, has a higher level of safety than the other two

gels. Ion-sensitive and pH-sensitive gels have been said to IRRITATE the eyes more, which could harm conjunctival cells [10]. Therefore. thermosensitive in situ gels, which undergo a sol-gel transition upon temperature change due to changes in the intermolecular interaction, are more promising for sustained ocular drug delivery [8]. There are various thermosensitive materials available with different gelling mechanisms and properties. PNIPAAm is a commonly used negative thermosensitive polymer that has recently aroused a lot of scientific curiosity due to its increasing solubility with decreasing temperature and ability to cause volume phase shift by generating hydrogen bonds[11]. PNIPAAm forms a stretched spiral elastic shape in the aqueous media; polymer molecules create hydrogen bonds with one another as a result of hydrogen bonding with water molecules. Its structure combines hydrophilic amide (-CONH-) groups and hydrophobic isopropyl (-CHCH3)2) side groups. PNIPAAm can be modified to create thermosensitive, in situ gels by copolymerizing with a variety of other hydrophobic or hydrophilic polymers. PNIPAAm has an LCST of 32 °C, which is somewhat lower than the average body temperature of 37 °C. The interactions between these groups and solvent molecules are the main causes of the temperature-responsive behaviour. As a result, as soon as the LCST hits body temperature, PNIPAAm transforms from a solution to a gel state. A larger LCST results from copolymerization with more hydrophilic monomers, which makes the polymer more hydrophilic and causes the polymer to interact with water in more important ways. In contrast, copolymerization with more hydrophobic monomers reduces LCST. PNIPAAm is suited for biomedical applications because of this characteristic, including controlled wound dressings, tissue engineering scaffolds, and drug delivery systems. Because the produced polymer matrices are brittle and unable to cling to the bottom of the vial, the poly(N-isopropylacrylamide) is not employed directly[12]. Due to the viscosity-increasing properties of natural polysaccharides , the grafted copolymer shows remarkable adhesion qualities. So to create graft copolymer, Tamarind seed polysaccharide is utilized.

Tamarind seed polysaccharide (TSP), Tamarind seed polysaccharide is isolated from *Tamarindus indica*seed kernel. Tamarind seed polysaccharide is actually a structural polymer that is abundant in cell wall of the higher plants. It is highly viscous, possesses a wide range of pH tolerance, and adhesive characteristics. As a result, it is extensively used as a stabilizer, thickening and gelling agent, and as a binder in the pharmaceutical and food sectors. Other essential features of TSP includenon-carcinogenicity, biocompatibility, mucoadhesive, and good drug-retaining capacity [13]. The different applications of TSP have been listed below (Table 1.1).

Table 1.1. Applications of TSP

Pharmaceutical applications	Reference
Bone tissue engineering	14
Controlled drug delivery	15
Gastro-retentive drug delivery	16
Flocculant for wastewater treatment	17

However, TSP suffers from several drawbacks such as unpleasant odor, dull color, fast degradability at higher temperature and uncontrolled rate of hydration [18.19]. The functionalgroups present in TSP have been chemically modified in order to change properties like thermal degradation, solubility, viscosity, and swelling [20]. Some chemically modified derivatives of TSP are given in Table 1.2.

Table 1.2. Chemically modified TSP

Chemical modification	Name of derivative	Characteristics obtained	Reference
Grafting	Polymethyl methacrylate-g-Tamarind seed polysacchride	Improved thermal stability Improved shelf life Controlled rate of hydration	21
Thiolation	Thiolated TSP	Improved mucoadhesive properties	22
Carboxymethylation	Carboxymethylated TSP	Increase in hydrophillicity of gum	23
Amination	Aminated TSP	Improved thermal stability Improved antimicrobial property Improved texture	24

2. Tamarind Seed Polysaccharide (TSP)

2.1 History

2.1.1 Source

Tamarind is a popularly known tree found in India. The scientific name is Tamarindus indica Linn. It is known as 'Indian date' and is also called Imli in Hindi. The tamarind tree is a dicotyledonous plant in the Leguminosae family [25]. Tamarind seed is one of the crucial by- products from the production process which is used for the pharmaceutical industry. Tamarindseed contains testa (20 to 30 %) and endosperm (70 to 80%). These can be also called seed coats and kernels. Plant polysaccharides are found in the endosperm and non-endospermic partsof seeds. Tamarind seed is the crude material for tamarind kernel powder manufacturing. Tamarind kernel polysaccharide (TKP), also known as tamarind gum, is derived from tamarindseed kernels [26]. Tamarind seed polysaccharides are obtained from tamarind kernel powder. There are several tamarind gum extraction processes reported from tamarind seed kernel powder. Within them, the first extraction was done by Rao et al in 1946 [27]. Then Rao and Srivastava in 1973 [28] and Nandi in 1975 [29] further reworked and modified the previous extraction process on the levels of the laboratory. Tamarind gum can be extracted using chemical and enzymatic methodsin most cases. Tamarind powder of seed (TSP) is steeped in boiling water and then filtered theextracted mucilage to separate in a chemical extraction technique. To make precipitate gum, the filtered mucilage part is mixed with an equivalent amount of ethanol or acetone. The precipitate obtained by extraction is then dried to form tamarind gum or tamarind seed polysaccharides [30]. Mixing tamarind kernel powder with ethanol and then reacting with an enzyme (protease) is the enzymatic extraction technique. It is centrifuged after being treated with protease enzymatically. The supernatant portion is taken. Then it is treated with ethanol to achieve tamarind kernel polysaccharide precipitation. Then precipitate is dried to form the gum [31].

2.1.2 Chemistry

TSP is amphiphilic because it has both hydrophilic and hydrophobic regions in its structure. The hydrophilic regions are the polar groups, such as xylose, galactose, and hydroxyl groups on glucose, that can interact with water molecules. The hydrophobic regions are the non-polar groups, such as the glucose backbone and the long hydrocarbon chains, that can interact with other non-polar molecules. Tamarind kernel polysaccharides are chemically neutral or non-ionic in nature. It is highly branched soluble hemicellulose structured polysaccharides. The molecular weight of TKP is close to about 50,000 Daltons or more than that. The backbone of the structure is composed ofa 1,4-linked β-D-Glucose backbone that's identical to cellulose. The chain of glucose or glucopyranose backbone is replaced with α-(1,6)-linked xylopyranose. This xylopyranose canbe further substituted by β-(1,2)-

linked residues of galactopyranosyl (Fig 1.1). Near about 80% of glucan backbones, are substituted by (1,6)-linked xylopyranose units and xylogalactopyranosyl units [32]. The xylopyranose units and subunits are used to be found linked up on the 2nd, 3rd, and 4th positions of the D-glucopyranosyl chain [33]. The monomer compositions are glucose, xylose, and galactose where percentage amounts are likely 55.4%, 28.4%, and 16.2%. So, the molecular ratio of the components (also written as glucose: xylose:galactose) is 2.8:2.25:1.0. As this type of composition, it is also called galactoTamarind seed polysacchride [34].

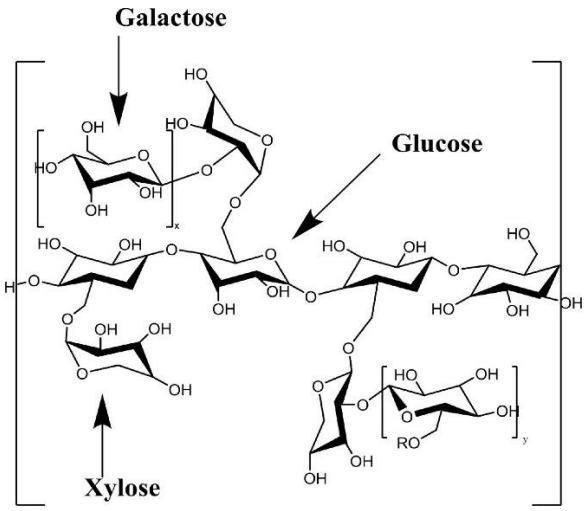

Fig 1.1 Structure of Tamarind Seed Polysaccharide [35]

2.1.3 General Properties

Native tamarind seed polysaccharide is water soluble like the rest of the polysaccharidenature but the whole amount is not hydrated. It possesses balanced nature of hydrophilic and hydrophobic. For the cellulosic type of backbone, the interchain interactions take place and are self-aggregated. The self-aggregated structure is shown like lateral fabricated strands of a single polysaccharide such as a worm-like chain, which is described in the so-called model of Kuhn's [36]. The stiffness is dependent on the number of aggregated strands. This type of nature is examined in SLS or static light scattering particles study. An extended stiffed structureof tamarind kernel polysaccharides can be obtained if it is substituted higher portion on the glucose chain. Then it will be occupied a larger volume [37]. TSP is insoluble in cold water but dispersed in warm water. In warm water, high viscosity gel is found. This produced gel hasa wide range of pH tolerance and adhesion property [38]. For the hydrophilic property, it swellsin the solution. This gel shows non-Newtonian and pseudoplastic rheological nature. It is not soluble in organic solvents such as ethanol, acetone, methanol, and ether just like other naturalgums [39]. It can withstand an acidic pH medium. Tamarind kernel polysaccharides may produce gels at both acidic and neutral pH media. It can make sugar-based gels that are viciousextremely. Tamarind gum is a biocompatible, biodegradable, non-irritant, and noncarcinogenic with hemostatic nature

polymer [40]. It has also been shown as a bioadhesive and a mucomimetic biopolymer. Tamarind gum's hepatoprotective, anti-inflammatory, and anti- diabetic properties have also been discovered [41]. It also has high flexible film-forming properties and tensile strength, as well as a high thermal stability and drug-holding capacity [42]. Tamarind gum, like other Tamarind seed polysacchrides, is not broken down by human digestive enzymes.It might be included in the dietary fiber portion of the diet. It is, however, fermented by intestinal microbes [43].

2.2 Tamarind seed polysaccharide-based modified drug delivery systems

The TSP-based modified drug delivery systems could be categorized as drug delivery systems based on either grafted TSP (chemically cross-linked) or coated TSP (physically cross-linked).

2.2.1 Grafted TSP-based drug delivery systems

Grafting of flexible synthetic polymer chains onto a rigid natural polysaccharide backbone effectively combines the essential properties of both the participating components to yield graft copolymer . Graft copolymerization has become a significant mode for development of advanced materials with improved functional properties, biodegradable, and reasonably shear stable . The graft copolymers are usually synthesized using conventional redox grafting technique , microwave irradiation , g-ray irradiation, and by using electron beams . As a tailormade material, graft copolymers find diverse applications in the delivery of therapeutic agents such as doxorubicin , Kaolin , metoprolol succinate , and curcumin [44].

Mahajan & Mahajan [45] have reported the development of (PLA) poly(L-lactic acid)-grafted TSP (PLA-g-TSP) micelles for pulmonary delivery of curcumin. PLA-g-TSP copolymer was synthesized by ring-opening polymerization reaction. The grafting of PLA chains onto TSP backbone was confirmed by FTIR and 1H NMR analysis. It was observed that the grafted copolymer (PLAg-TSP) forms micelles at a critical micelle concentration of 0.015 w% with an average micelle diameter of ∼102 nm, as estimated by dynamic light scattering. Curcumin-loaded micelles exhibited a zeta potential value of -18.2 mV, drug loading ∼69%, and entrapment efficiency of ∼96%. The in vitro drug release was found to be sustained over a period of 5 h. The in vitro assessment also demonstrated the suitability of micelles as dry powder for inhalation (DPI). In vivo studies executed on male Wistar rat model revealed significant improvement in the bioavailability of curcumin after pulmonary administration of micelles as DPI. In conclusion, the authors have stated that polymeric micelles based on a newly synthesized copolymer (PLA-g-TSP) could be a potential carrier for pulmonary delivery of curcumin.

2.2.2 Coated TSP-based drug delivery systems

It has been reported that the modification of the surface of particles by coating with polysaccharides is a potential strategy to control stabilization of colloidal particles like bacterial cellulose nanocrystals

or water-in-water emulsions [44].

Liu et al. [46] also reported the application of TSP as a targeting ligand for the intracellular delivery of 10-hydroxycamptothecin (HCPT) to the hepatoma after modification of the surface of nanostructured lipid carriers (NLCs) with TSP. HCPT is widely used in clinical therapy against hepatoma however; its chemotherapy is strongly obstructed by the emergence of multidrug resistance (MDR). The authors have prepared NLCs coated with TSP. TSP consists of side chains with galactose residues, a terminal moiety that can be used to target HCPT to hepatoma. Galactose is a ligand for the asialoglycoprotein receptor (ASGPR), which is a transmembrane receptor and locates mainly on the sinusoidal membrane of hepatocytes. TSP can serve as hepatocyte-targeting carrier due to the specific ligand-receptor interactions between galactose moieties of TSP and ASGPR. The therapeutic potential of TSP-NLC/HCPT was investigated on HepG2/HCPT and also on human tumor xenograft nude mouse model (implanted with HepG2/HCPT cells). TSPNLC/HCPT indicated superior cytotoxicity against the drug resistant HepG2/HCPT cells and also generated a higher therapeutic efficacy in vivo. TSP-NLC/HCPT elaborated a promising potential as a drug delivery system to overcome MDR of HepG2/HCPT cells. In conclusion, the outcomes of this investigation suggested that the HCPT-loaded NLCs coated with TSP represent a potential carrier for intracellular delivery to the hepatoma and reverse the drug resistance of HepG2 cells. Finally, TSP could potentially be exploited as a specific targeting ligand for liver tissues.

2.2.3 Semi-synthetic derivatives of TSP

The schematic diagram illustrating the possible chemical modifications of TSP is displayed as Fig. 1.2. In general, three semi-synthetic derivatives of TSP have been synthesized and utilized for drug delivery applications viz. thiolated TSP, carboxymethylated TSP, and aminated TSP.

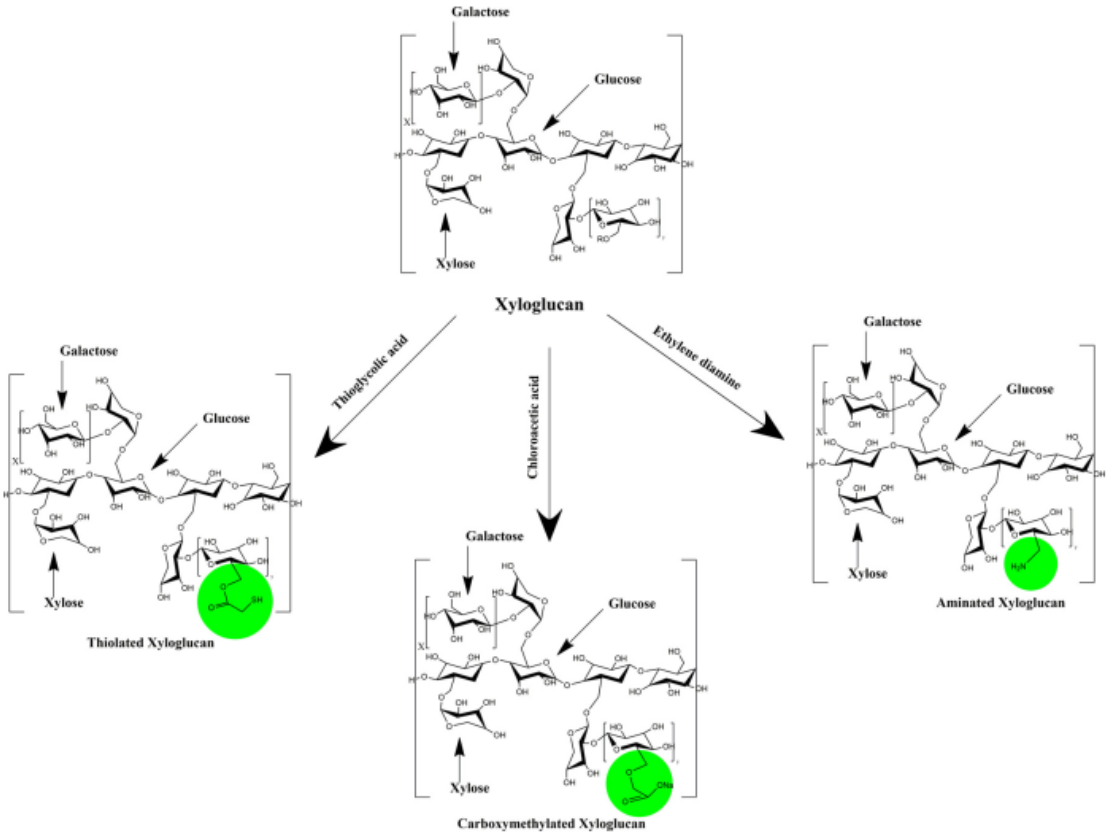

Fig. 1.2 Scheme of modification of TSP to its thiolated, carboxymethylated and aminated derivative

2.2.4 Thiolated TSP

Thiomers are the polymers modified by incorporating the thiol functional groups in the backbone of the native polymeric chains either by substitution reaction or by simple oxidation reaction. It has been proposed that the incorporation of thiol groups results in the enhancement of mucoadhesion potential of the modified polymer by 2–140 folds over native polymer . Thiolation of the natural polymers is an important strategy adapted by many researchers in order to improve the mucoadhesive and cohesive properties of the polymers . The mechanism underlying the mucoadhesion of thiolated polymers is based on thiol/disulfide exchange reaction and on an oxidation process between the reactive thiol groups of the thiomers and cysteine-rich subdomains of the mucus glycoprotein . In addition to the enhanced mucoadhesiveness, thiomers have also been found to enhance the oral permeability of therapeutic proteins and peptides and exhibit in situ gelling potential . Recently, thiolation of TSP has been carried out in order to investigate the improved mucoadhesion potential , in situ gelation , and improved permeability of thiolated derivative over native TSP[44].

Kaur et al. [47] carried out the thiolation of the TSP by esterification with thioglycolic acid. The thiolation was confirmed by the FTIR analysis. DSC and XRD analysis revealed increased crystallinity of thiolated TSP over native TSP. Comparison of mucoadhesion potential using tensile test on texture profile analyzer indicated that the thiolated TSP Exhibits 6.85 fold greater

mucoadhesive strength compared to native TSP. To further explore the mucoadhesive strength, Carbopol-based metronidazole gels containing TSP and thiolated TSP were formulated and compared with a commercial formulation of metronidazole gel. It was revealed that the gel containing thiolated TSP exhibited higher mucoadhesion than the gel containing native TSP. The authors concluded that the promising mucoadhesive properties of the thiolated TSP warrant its application in the formulation of bioadhesive drug delivery systems.

2.2.5 Carboxymethylated TSP

TSP is a water-soluble polysaccharide, but due to the brawbacks associated with TSP like unpleasant odor (due to presence of fats), dull color, and fast biodegradability, it was found wanting in a number of speciality end-use properties, particularly in food and drug delivery applications . In the context of the above mentioned need, TSP has been modified by chemical treatment with various functional groups like acetyl, hydroxyalkyl, and carboxymethyl . Carboxymethylation of TSP imparts anionic trait to the polymer. Derivatization of TSP with a carboxymethyl group (twostep reaction using sodium hydroxide and monochloroacetic acid) results in the disordered structural symmetry. This derivatization has exposed the polysaccharide network for hydration resulting in higher viscosity and lower biodegradability (carboxymethyl group makes the molecule resistant to enzymatic attack), thereby enhancing its shelf life. Carboxymethyl TSP, thus, presents a potential opportunity in pharmaceutical nanotechnology as a novel carrier of drugs in controlled release formulations due to its enhanced swelling ability, improved solubility in cold water, and improved stability[44] .

Kaur et al. [48] reported the preparation and evaluation of nanoparticles (NPs) based on carboxymethylated TSP for ocular delivery of tropicamide. The NPs were optimized using three-levels, two-factor central composite design. It has been found that the concentration of polymer (carboxymethyl TSP) and cross linking agent (calcium chloride) has a synergistic effect on the particle size and encapsulation efficiency of NPs. The ex vivo corneal permeation studies executed on optimized tropicamide-loaded NP formulation across the isolated goat cornea was comparable to the aqueous solution of tropicamide. However, the results of bioadhesion analysis using mucus glycoprotein assay revealed that the carboxymethyl TSP-based NPs exhibited higher mucoadhesiveness. In addition, the in vitro ocular tolerance test carried out on the fertilized chicken eggs (HET-CAM study) indicated the non-irritant nature of fabricated NPs. These findings suggested the suitability of carboxymethyl TSP-based NPs for ocular administration of tropicamide.

2.2.6 Aminated TSP

Natural, non-toxic, and water-soluble polysaccharides are finding numerous applications in foods, textiles, paints, cosmetics, and pharmaceuticals which utilize its broad range of functional properties. Chemical derivatization reactions are used to alter or improve the functional properties of these

intractable, but inexpensive polysaccharides. It has been established that the non-ionic polymers exhibits poor mucoadhesive/bioadhesive strength than the ionic polymers . Cationic polysaccharides find wide applications in drug and gene delivery. It also finds applicability in new areas like biotronics and fluorescent labeling applications.

Simi & Abraham [49] synthesized a cationic derivative of TSP by functionalizing amino groups on the backbone of TSP. The derivatization was confirmed by FTIR and NMR analyses. Amination of TSP with ethylene diamine in aqueous medium yielded irreversible hydrogels with blue fluorescence characteristics. Aminated TSP form self-assembled nanoparticles at very low concentration (0.2% w/v) whereas at high concentration (7% w/v), it forms a strong hydrogel. The aminated TSP had same solubility as TSP and also exhibited good thermal properties. The aminated TSP showed better antimicrobial activity as compared to chitosan. In conclusion, the biodegradability, biocompatibility, strong gelling behavior, and blue fluorescence characteristics are very promising to explore the utility of aminated TSP in pharmaceutical, biomedical, biotronics, and fluorescence labeling applications.

Chemical grafting method using the potassium per sulphate and ascorbic acid redox pair is ideal. The physical characterization of the grafted tamarind seed polysaccharide shows no drop in viscosity with storage and a regulated rate of hydration (GTS).

2.3 Phase transition for PNIPAAm

Poly(N-isopropyl-acrylamide) and other N-substituted acrylamide polymers (PNIPAAM) have balanced hydrophilic and hydrophobic regions below LCST[11] The gel-polymer/water system' s total energy is lowered due to hydrophobic polymers enveloped by water molecules below LCST. The salvation and transition capacity of PNIPAAm in cold water increases when the temperature is raised off its LCST (LCST-32-34 °C), leading to the "coil to globule" of the polymeric chain's (CG) transition. The CG transition is in charge of phase inversion into rich layers. Polymeric/water phases further exhibit volume phase transition (VPT). A loss in entropy of water molecules enveloping the hydrophilic polymeric chain is counter balanced by an increase in enthalpy owing to hydrogen bonding between the hydroxyl groups surrounding the polymeric chain’s hydrophobic sections. Hydrophobic hydration is a process that allows a hydrophobic polymer to stay hydrated in an aqueous environment. If the temperature is increased above LCST, water molecules leave the polymer chain and form a globule structure. As a result, the PNIPAAm-polymer is hydrated, and a definite volume phase transition is observed. The phase separation initially occurs due to PNIPAAm molecule incorporation into larger aggregates via several mechanisms and factors, e.g., dewetting caused by solvent fluctuations, cooperative hydration, the aqueous medium's energy state, endothermic heat, precipitation polymerization, etc. The hydrogen bond between water molecules and PNIPAAm is weaker due to the temperature rising above LCST, leading to the formation of an unstable solution.

2.4 The role of the hydrogen bonding interactions

At a cloud point, PNIPAAm exhibits a unique volume phase transition from a hydrated state called a hydrophilic state with an expanded structure to a shrunken dehydrated state called a collapsed structure [50]. The presence of hydrophilic and hydrophobic groups inside the neutral polymer is responsible for this reversible sol-gel behavior of PNIPAAm homopolymer in water solutions. The reversibility of the hydrophilic/hydrophobic states occurs by varying the temperature below or above the LCST value (32 °C) (Fig l.3). The LCST is the temperature above which the gel becomes insoluble in an aqueous environment. LCST depends on the Critical gel concentration (CGC). Then, at its CGC, solvated PNIPAAm molecules will exhibit aqueous insolubility upon heating above the LCST. The LCST is mainly dependent on the hydrogen bonding between water molecules and the structure of functional monomer units of PNIPAAm polymer; ie., N-H and C-O linkages. Thus, the incorporation of hydrophilic units typically increases the volume-phase transition temperature (VPTT), whereas the addition of hydrophobic units has the opposite effect.

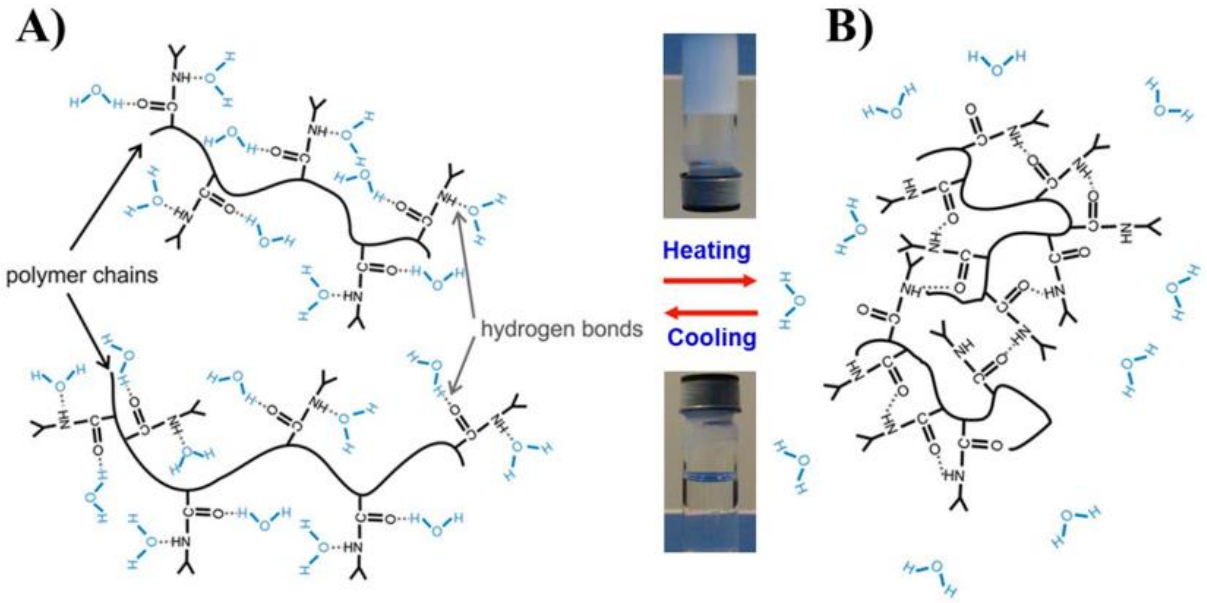

Fig 1.3 Difference in hydrogen bonding before and after gel formation

2.5 Biocompatibility and biodegradability of PNIPAAm based in situ gel

Polymers' physical and chemical characteristics (such as melting point, glass transition temperature, crystallinity, storage modulus, etc.) have an impact on how biodegradable they are. The use of PNIPAAm hydrogel in clinical medicine has been constrained by its poor biodegradability. By adding various biodegradable monomers and/or crosslinkers or natural polymers like poly(amino acids), polysaccharides, and proteins, as well as synthetic polymers like poly(esters), poly(caprolactone), and poly(ethylene glycol) (PEG), various methods for the preparation of biodegradable PNIPAAm-based hydrogels are being investigated. Both biodegradability and biocompatibility are necessary features

depending on the application target (for example, in medication delivery and cell encapsulation). Animal cells are extremely biocompatible with PNIPAAm. In situ PNIPAAm-gelatin gel production was documented by Matsuda and colleagues in rat subcutaneous tissue. They histologically studied the rat fibroblast development for up to 12 weeks following organogel injection. The inflammatory response manifested at the time of injection but went away after two weeks, and the fibroblasts within the gel expanded and multiplied. This study establishes the viability of implanting PNIPAAm- gelatin gel as a cell scaffold in vivo.

2.6 In situ gel

The word in-situ is derived from a Latin term that means "in its original place or in position" [8]. When exposed to physiological conditions including pH, temperature, and ionic strength in the environment, in situ gel undergoes a phase shift from liquid to semi solid gel. When injected into the eye, in-situ forming ophthalmic gels are liquids that undergo rapid gelation in the eye's cul-de-sac in reaction to environmental changes to generate viscoelastic gels (Fig 1.4) [51]. lastly release the drug slowly under physiological conditions. Additionally, the in-situ gel's residence period will be prolonged, and the drug will be given gradually. These factors increase bioavailability, reduce systemic absorption, and need fewer frequent doses, which improves patient compliance. In-situ gelling devices have also demonstrated several additional potential benefits such an easy manufacturing process, convenience of administration, and delivery of a precise dose.

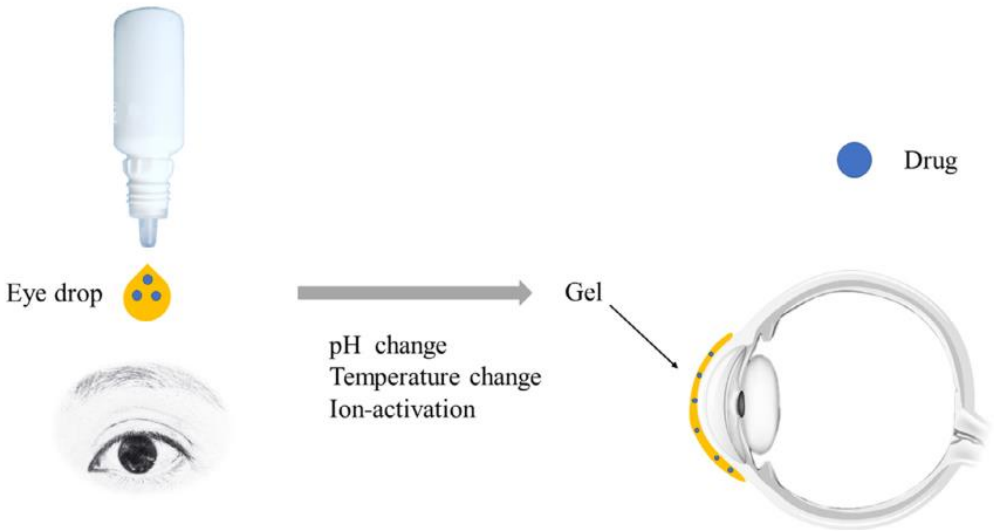

Fig. 1.4 In-situ ophthalmic gel

2.6.1 Advantages of in situ gels

- Less blurred vision as compared to ointment.
- Decreased nasolacrimal drainage of the drug which may cause undesirable side effects due to systemic absorption (i.e. reduced systemic side effects).The possibility of administering accurate and reproducible quantities, in contrast to already gelled formulations and moreover promoting precorneal

retention.

- Sustained, Prolonged drug release and maintaining relatively constant plasma profile.
- Reduced frequency of applications hence improved patient compliance and comfort.
- Generally more comfortable than insoluble or soluble insertion.
- Improved local bioavailability due to increased precorneal residence time and absorption.
- Its production is less complex and thus lowers the investment and manufacturing cost [52]

2.6.2 Classification of in situ gel polymers

Based on their origin, polymers are classified or the mechanism of gelation. According to a source in situ, gelling systems classified into two types:

i. Natural polymers (E. g., Alginic acid, Carrageenan, chitosan, Guar gum, gellan gum, pectin, sodium hyaluronate, xanthan gum, xyloglucan, etc.)

ii. Synthetic or semi-synthetic polymers (E. g., Cellulose acetate phthalate, hydroxypropyl methylcellulose, methylcellulose, polyacrylic acid, poly (lactic-co-glycolic acid, poloxamers) [53].

2.7 Temperature responsive in situ gel

Based on the stimuli, in situ gels can be divided into ion-activated, thermosensitive and pH-sensitive types [9]. The thermosensitive in situ gel has superior safety than the other two in situ gels. Ion-sensitive and pH-sensitive gels have reportedly been found to irritate the eyes more, which can harm conjunctival cells. For prolonged ocular drug administration, thermosensitive in situ gels are therefore more promising since they go through a sol-gel transition when heated or cooled due to changes in the intermolecular interaction (Fig 1.5). The oldest, most well-studied, and most popular kind of stimuli-responsive gel is the temperature sensitive ophthalmic in-situ gel [51]. It may be applied to the eye in liquid form with ease and precision without irritating the eye or impairing vision. The gel is created at precorneal temperature (35 °C) to withstand the dilution of injected medication by lachrymal fluid without causing rapid precorneal elimination. A good thermo- responsive ocular in-situ gel has been advised to have the gelation temperature above room temperature and go through the gel-sol transition at a pre-corneal temperature to avoid having to store it in the refrigerator before use, which could occasionally cause eye irritation due to its cold nature.

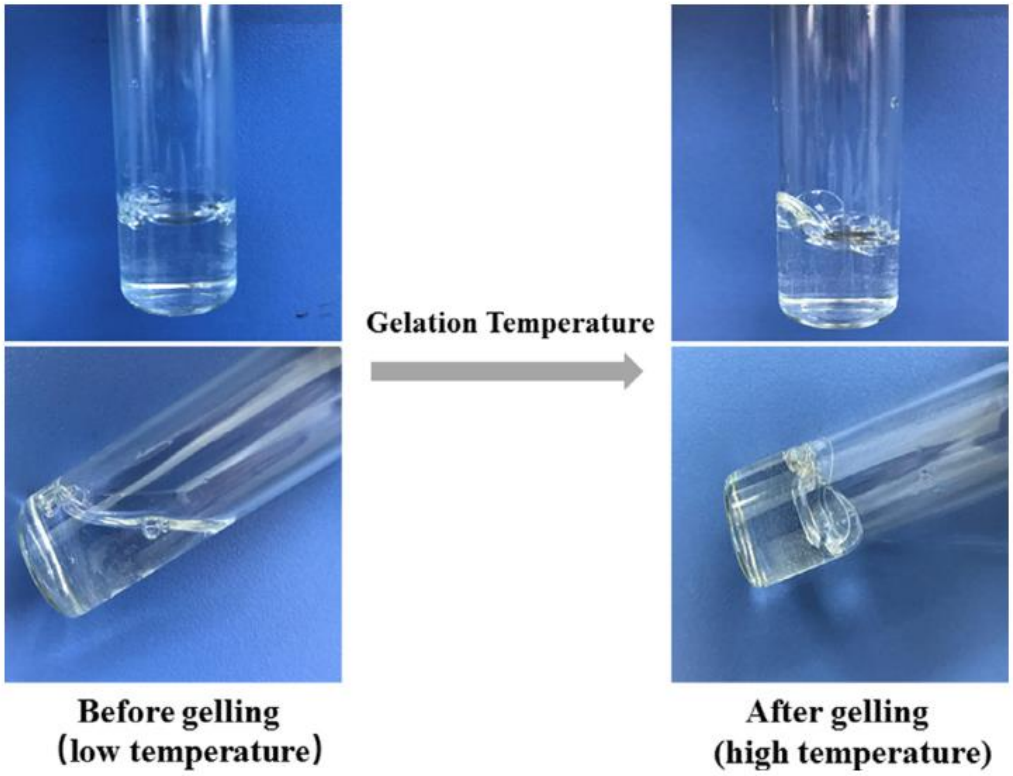

Fig. 1.5 Temperature responsive in-situ gel

2.8 Glaucoma

The angle of the eye is the junction between the iris and cornea, where the trabecular meshwork drains aqueous humor from the anterior chamber of the eye *(Figure 1)*. In POAG, the angle remains open as the trabecular meshwork is unblocked by iris tissue. Intraocular pressure is transmitted to the axons of retinal ganglion cells at the optic nerve as mechanical stress, leading to cell death.However, about 50% of patients with glaucoma have intraocular pressure within the so-called “normal” range of 10 to 21 mm Hg at diagnosis.Only after 30% of retinal ganglion cells have been lost are visual field defects present on perimetric testing (Fig 1.6) [54].

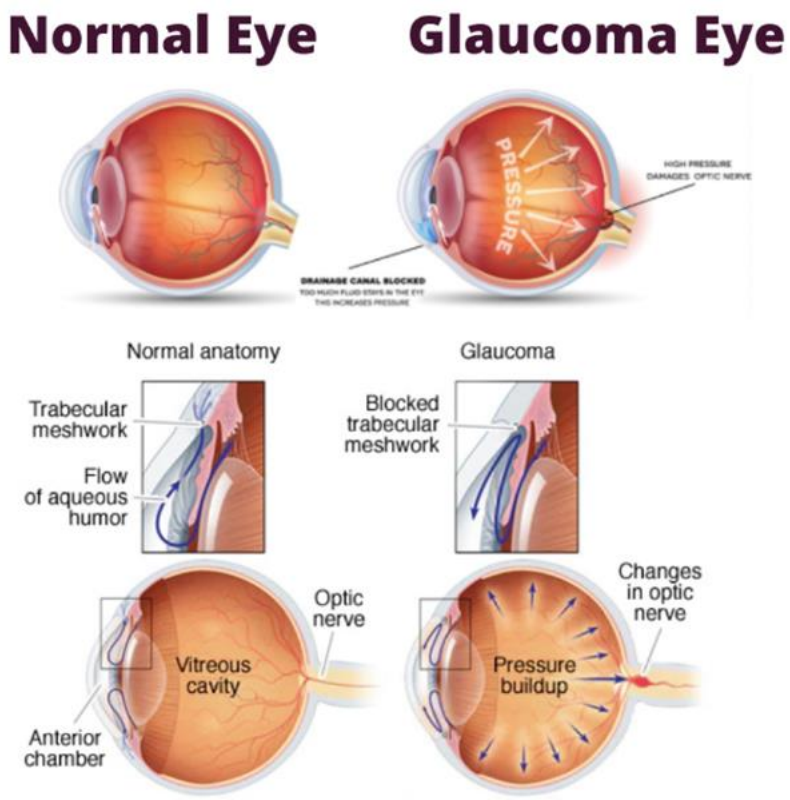

Fig. 1.6 Comparison between normal eye and glaucoma eye

2.9 Objective of the work

The objective of the present work was to develop a temperature-sensitive in situ gelling ophthalmic delivery system of Dorzolamide HCL. Dorzolamide hydrochloride is a carbonic anhydrase inhibitor used in the treatment of glaucoma. Carbonic anhydrase inhibitors reduce IOP by decreasing aqueous humor secretion through the inhibition of carbonic anhydrase isoenzyme II in the ciliary processes[2]. Dorzolamide hydrochloride should be applied topically to the affected eye every three hours, or hourly in case of severe infection. The recommended dosage for this is 1-2 drops of a 2% w/v solution. A solution of Poly(NIPAAm-g-TSP) which would gel when instilled into the eye provides sustained release of the drug treatment in glaucoma.

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CHAPTER 2
LITERETURE REVIEW

2. LITERATURE REVIEW

Goyal et. al. developed an graft copolymer of acrylamide (AA) onto TKP in an aqueous medium using a ceric ammonium nitrate (CAN)-nitric acid initiation system [1]. The reaction conditions were optimized for grafting with respect to the effect of the concentrations of CAN, nitric acid, TKP, AA, time, and reaction temperature. The maximum percentage grafting (%G) and percentage grafting efficiency (%GE) were found to be 231.45 and 93.66%, respectively.

Ghosh et. al. produced novel biodegradable polymeric flocculants by conventional redox grafting of acrylamide to tamarind kernel polysaccharide [2]. The graft copolymers were characterized by viscometry, elemental analysis, molecular weight determination using SLS analysis, and NMR spectroscopy. The flocculation efficiency of the grafting products in kaolin suspension, municipal sewage wastewater and textile industry wastewater was primarily dependent on the length of the grafted polyacrylamide chain. The flocculant obtained by microwave-assisted grafting method was superior to TKP and polyacrylamide-based commercial flocculant (Rishfloc 226 LV) in flocculation tests.

Nandi et. al. synthesied polyacrylamide-grafted-tamarind seed gum (PAAm-g-TSG) employing free radical method assisted with microwave where cerric (IV) ammonium nitrate (CAN) was used as free radical initiator [3]. Effects of monomer, CAN and microwave irradiation time (MW) on grafting were studied. Various grafting indicators such as % grafting (%G), % grafting efficiency (%GE) and % conversion (%Cn) were calculated. MW and CAN exhibited major contributions to the synthesis.

Mehra et. al. formulated in-situ gels for the delivery of pilocarpine with the help of alginate, tamarind gum and chitosan [4]. In-vivo miotic test was performed along with bioadhesive strength, in-vitro drug release, drug content uniformity and gelation studies. All of the formulations were found to satisfy the desirable conditions of gelation, content uniformity and bioadhesion. The best drug release in slow rate was observed in case of the formulation prepared with tamarind gum. 25% of the drug was released in the initial hours while the rest 80% was released in the next 12 hours. In-vivo miotic studies also showed best results with tamarind gum.

Cao et. al. investigated novel copolymer, poly(N-isopropylacrylamide)–chitosan (PNIPAAm–CS), for its thermosensitive in situ gel-forming properties and potential utilization for ocular drug delivery [5]. The thermal sensitivity and low critical solution temperature (LCST) were determined by the cloud point method. PNIPAAm–CS had a LCST of 32 °C, which is close to the surface temperature of the eye. The in vivo ocular pharmacokinetics of timolol maleate in PNIPAAm–CS solution were evaluated and compared to that in conventional eye drop solution

by using rabbits according to the microdialysis method. . Furthermore, the PNIPAAm–CS gel-forming solution of timolol maleate had a stronger capacity to reduce the intra-ocular pressure (IOP) than that of the conventional eye drop of same concentration over a period of 12 h.

Giri et. al. synthesized polyacrylamide (PAAm) grafted locust bean gum (LBG) via the conventional method using ceric ammonium nitrate (CAN) as free radical initiator to improve flocculation efficiency [6]. Found that The graft copolymer shows swelling-deswelling behavior in buffer solutions with pH 2.0 and 9.0. The intrinsic viscosity of LBG improved significantly on grafting of polyacrylamide chains. Grafted copolymer gives better performance by showing lower turbidity than LBG. No mortality of the animal was observed during 14 days after treatment with graft copolymer.

Zakerikhoob et. al. developed curcumin-incorporated crosslinked sodium alginate-g-poly (N-isopropyl acrylamide) thermo-responsive hydrogel as an in-situ forming injectable dressing for wound healing [7].

Kataria et. al. developd an efficient in situ gel forming system of dorzolamide for the treatment of glaucoma [8]. The in situ gel was formulated using alginate and hydroxyl propyl methyl cellulose (HPMC) polymer combination. The in situ alginate/HPMC gel forming system could be a potential approach for Dorzolamide formulation to enhance ocular bioavailability for the treatment of glaucoma, ultimately improving the patient compliance.

Duan et. al. developed and characterized pH-responsive and biocompatible nanogels as a tumor-targeting drug delivery system [9]. The nanogels were self-assembled from chitosanbased copolymers, chitosan-graft-poly(N-isopropylacrylamide) (CS-g-PNIPAm). The copolymers were synthesized via free radical copolymerization and characterized for their chemical structure by FT-IR and 1H NMR. These copolymers could be efficiently loaded with oridonin (ORI) and the characteristics of ORI-loaded nanogels were evaluated. Drug release researches indicated that the ORI-loaded nanogels displayed pH-dependent release behaviors.

Kajjari et. al. prepared and characterized novel pH- and thermo-responsive blend hydrogel microspheres of sodium alginates (Na Alg) and poly (N-isopropylacrylamide)(PNIPAAm)-grafted-guar gum (GG) i.e., PNIPAAm-g-GG by emulsion cross-linking method using glutaraldehyde (GA) as a cross-linker [10]. Isoniazid (INZ) was chosen as the model antituberculosis drug to achieve encapsulation up to 62%. INZ has a plasma half-life of 1.5 h, whose release was extended up to 12 h.

Giri et. al. developed polyacrylamide (PAAm) grafted gellan gum (GG) using microwave irradiation [11]. The grafting condition was optimized by varying the microwave power, exposure time, and concentrations of initiator, monomer, and GG. The flocculation

characteristics of grafted and ungrafted polysaccharides have been evaluated in coal (coking and noncoking) suspensions. Graft copolymer shows better flocculation efficacy compared to the base polysaccharides

Sandeep et. al. formulated the thermosensitive ophthalmic in-situ gels of dorzolamide HCl for the treatment of glaucoma [12]. The results of the current study conclude that Dorzolamide HCl in-situ gel is a better alternative approach providing sustained drug release for the management of glaucoma.

Katiyar et. al. formulated the in situ gel of chitosan nanoparticles to enhance the bioavailability and efficacy of dorzolamide in the glaucoma treatment [13]. Optimized nanoparticles were spherical in shape (particle size: 164 nm) with a loading efficiency of 98.1%. The ex vivo release of the optimized in situ gel nanoparticle formulation showed a sustained drug release as compared to marketed formulation. The gamma scintigraphic study of prepared in situ nanoparticle gel showed good corneal retention compared to marketed formulation. HET-CAM assay of the prepared formulation scored 0.33 in 5 min which indicates the non-irritant property of the formulation. Thus in situ gel of dorzolamide hydrochloride loaded nanoparticles offers a more intensive treatment of glaucoma and a better patient compliance as it requires fewer applications per day compared to conventional eye drops.

Miguel et. al. evaluated the effect of topical dorzolamide on the intrastromal corneal pressure (ICP) in rabbit corneas in vivo [14]. This is an interventional prospective study. Topical dorzolamide was applied to 7 eyes of 7 male New Zealand rabbits 3 times daily for 3 consecutive days. The ICP changes were recorded with a pressure transducer connected to the midperipheral cornea. The ICP was measured in the same manner in 7 eyes of 7 male New Zealand rabbits that were treated with artificial tears (control group). The ICP values averaged 26.2 6 3.2, 210 6 5.8, and 212.5 6 8.7 mm Hg at 15, 30, and 45 minutes in the control group, respectively. In the dorzolamide-treated eyes, the ICP readings were 1.8 6 3.4, 20.28 6 4.3, and 21.8 6 5.3 mm Hg at the same time points, respectively. The differences in the ICP between both groups were significantly different at all time points (P = 0.004, P = 0.005, and P = 0.02)

Mishra et. al. performed the grafting of polyacrylonitrile (PAN) onto xyloglucan (XG) bythe ceric ammonium nitrate/HNO3 (CAN) initiator under N_2 atmosphere [15]. XG-g-PAN exhibited a crystalline behavior in comparison to the pure polysaccharide which showed amorphous nature. The obtained copolymer had better thermal properties than the pure polysaccharide.

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CHAPTER 3
MATERIALS & METHODS

2. Materials and methods

2.1 Materials

DRZ was kindly gifted by APDM Pharmaceutics Pvt. Ltd., Gujrat, India. NIPAAm was obtained from SRL Pvt. Ltd. Maharashtra, India. TSP is obtained as a gift sample from Hindustan Gum & Chemical Limited, Bhiwani, India). Acetone and methanol were purchased commercially from Merck Specialties Pvt. Ltd., Mumbai, India. All reagents used in the study were of analytical grade.

2.2 Synthesis of graft copolymer

The requisite quantity of TSP was added to a beaker containing water (10 ml) and heated at 60°C for 3 h. The mixture was then left overnight to ensure complete hydration. Separately, the NIPAAm was dissolved in water (2 ml). Both solutions were combined in a reaction vessel and mixed thoroughly for 1 hour using a magnetic stirrer at 26-27°C with continuous nitrogen purging. Following this, 0.01-0.02 (M) of ceric ammonium nitrate (CAN) was dissolved in 0.1-0.4 (M) of nitric acid and added to the vessel under continuous stirring. The mixture was then maintained for 4 h under continuous nitrogen purging [1]. Upon completion of the reaction, an excess of acetone was added to precipitate the graft copolymer. The precipitate was collected and washed three times with a methanol-water mixture in a 70:30 ratio to remove the homopolymer [2]. After that, the product was dried at 40°C until it reached a constant weight. The percentage grafting (% G) and percentage grafting efficiency (% GE) were determined utilizing the equations given below [3].

$$\% G = \frac{(P_g - P_c)}{P_c} \times 100 \quad \text{.....(I)}$$

$$\% GE = \frac{(P_g - P_c)}{P_m} \times 100 \quad \text{.....(II)}$$

Where P_g, P_c, and P_m denote the weight of the graft copolymer, TSP, and NIPAAm respectively.

2.3 Fourier transform infrared (FTIR) spectroscopy study

The FTIR spectra of TSP and NIPAAm-g-TSP were analyzed using an FTIR spectrophotometer **(Rx 1, Perkin Elmer, UK)** at 400-4000 cm^{-1} [4]. The preparation of samples for FTIR analysis involved thoroughly mixing the powdered samples with potassium bromide (KBr). The mixture was then subjected to high pressure using a hydraulic press to form transparent pellets suitable for FTIR measurement.

2.4 Nuclear magnetic resonance (NMR) spectroscopy study

The 13C solid-state NMR spectroscopy of TSP and NIPAAm-g-TSP was carefully conducted to confirm the grafting of TSP [5]. NMR spectra were acquired using a JEOL ECX 500 MHz NMR spectrometer, operating at a spinning frequency of 10 kHz. The powdered samples were placed

in a zirconium rotor with a diameter of 3.2 mm and spun at 10 kHz. The reference material used was tetramethyl silane.

2.5 Preparation of in situ gels

DRZ (2% w/v) was first added to distilled water in a test tube with continuous stirring to ensure that the drug was fully dissolved. Then a weighted quantity of grafted copolymer was carefully added to the drug solution. The mixture was continuously stirred to ensure that the grafted copolymer was completely dissolved. Lastly, benzalkonium chloride (0.02 % w/v) was incorporated into the preparation.

2.6 Clarity test

A careful visual inspection was used to assess the solutions' clarity. This inspection was carried out under both black and white backgrounds. By using contrasting backgrounds, any potential impurities or cloudiness in the solutions could be easily detected [6].

2.7 pH determination

The pH was determined immediately after preparation with a digital pH meter. Each formulation's pH was measured three times to verify the uniformity and reproducibility of the results.

2.8 Determination of gelling capacity

The gelling capacity was evaluated through a method involving the placement of a drop of the formulation into a beaker containing simulated tear fluid (STF) at 37°C [7]. Visual assessment was conducted to observe the time required for gel formation, noting both the time required for gelation to occur and time required to dissolve the gel.

2.9 Determination of gelling temperature

A standardized test tube inversion method was used to assess the gelling temperature of the formulations [8]. The formulation (2 ml) containing a test tube was placed in a beaker containing water. Successively, the temperature was raised slowly from 30 to 40˚C. The temperature at which the solution inside the test tube ceased moving was recorded as the gelling temperature.

2.10 Determination of phase transition temperature and degree of transparency

The phase transition temperature of the in situ gel forming solution was determined using a UV visible spectrophotometer. The gel forming solution was converted into milky white gel at phase transition temperature. The gel forming solution (1mg/ml) was taken in cuvettes and heated from 30 to 40˚C. The transmittance measurements were recorded at different temperatures at 600nm. This wavelength was selected because the range of 550-600 nm corresponds to the most sensitive colour in daylight, and 600 nm is 2.7 times more sensitive

than other colours [9-10].

2.11 Determination of viscosity

The rheological studies were conducted using a Brookfield viscometer [11]. The viscosity of the optimized formulation was measured under two different conditions: at varying temperatures with a fixed rpm and at varying rpms with a fixed temperature. 50 mL of the formulation was taken in a beaker. The number 21 spindle was immersed up to the designated mark inside the beaker. Formulation viscosity was determined at different rpm maintaining a fixed temperature of 37°C. The experimental setup was carefully arranged to ensure that the temperature could be maintained at a fixed value. Subsequently, the temperature was gradually changed from 25°C to 41°C at a fixed rpm of 30.

2.13 In vitro release studies

The in vitro dissolution study was carried out using a test tube with a diameter of 2 cm, both ends of which were open [12-13]. A dialysis membrane, which had been soaked overnight in STF, was securely tied to one end of the test tube. This end was then placed into a beaker containing 50 mL of STF. The setup was arranged such that the dialysis membrane just touched the surface of the dissolution medium. 1 ml of the formulation was carefully introduced into the open end of the test tube. The entire assembly was placed onto a magnetic stirrer and rotate the solution at a constant speed of 50 rpm at 37˚C. 1 mL of the medium was withdrawn at specified intervals from the beaker and replaced with freshly prepared STF to maintain a sink condition. The withdrawn samples were appropriately diluted, and their absorbances were measured at 256 nm using UV-visible spectrophotometer (UV 2450, Shimadzu, Japan).

2.12 Ocular irritation study on goat cornea

The study was performed using goat cornea obtained from a local slaughterhouse. The cornea was washed with normal saline. Then incubated separately with normal saline (negative control), 0.1% sodium dodecyl sulfate (SDS) solution (positive control), and optimized in situ gel formulation. After a 5-hour incubation period, the cornea was thoroughly washed with phosphate-buffered saline (PBS) to remove any residual formulation and immediately fixed in a 10% (w/w) formalin solution to preserve tissue structure. The fixed tissue was then subjected to a dehydration process using an alcohol. Subsequently, the tissue was embedded in melted paraffin to solidify and form a block, facilitating the preparation of thin cross-sectional slices. These sections were stained with haematoxylin and eosin, allowing microscopic examination of cellular and tissue modifications under a digital microscope [14].

2.14 Ex vivo permeation study

Ex vivo drug permeation studies were performed on freshly removed goat corneas [15]. The

cornea was securely attached to one side of a 2 cm inverted tube. The tube's corneal end was dipped in a beaker containing 50 mL of STF. This beaker was put on a magnetic stirrer and the solution was continually stirred at 50 rpm and 37OC. The in-situ gel forming solution (1 ml) was introduced into one side of the opened tube. 1 mL of the medium was withdrawn at specified intervals from the beaker and replaced with freshly prepared STF to maintain a sink condition. The withdrawn samples were appropriately diluted and their absorbances were measured at 256 nm using a UV-visible spectrophotometer.

2.15 Determination of Isotonicity

Blood was collected in an EDTA vial to prevent clotting. A few drops of the formulation were placed into a china dish, followed by the addition of a few drops of blood. The mixture was gently shaken to ensure thorough mixing of the blood and the formulation. Next, a blood sample was drawn from the china dish into a red blood cell (RBC) pipette up to the 0.5 mark, and then further diluted with RBC diluting fluid. A drop of this diluted sample was placed on a glass slide and covered with a cover slip. The glass slide was then carefully placed on the mechanical stage of a microscope. Observations were made using a 45x magnification lens to evaluate the tonicity of the formulation. The effect on the red blood cells (RBCs) was checked for signs of crenation (shrinking), swelling, or bursting [16].

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CHAPTER 4

RESULTS, DISCUSSION & CONCLUSION

The temperature-sensitive NIPAAm-grafted TSP was synthesized using CAN as an initiator by the free radical graft copolymerization technique. In the existence of nitric acid, the ceric ion (CeIV) attacks the TSP backbone chain, forming a TSP-ceric complex. Subsequently, the CeIV ions within the complex are reduced to cerous ions (CeIII), leading to the generation of a free radical at either the C2 or C3 position on the backbone (Fig 4.1) [1]. In the initiation step, a free radical present in the polymeric backbone reacts with a monomer containing stable molecules to form a new reactive species. During the propagation step, this newly formed species can further react with additional stable molecules, leading to a chain reaction that builds up the polymer chain in the case of polymerization. Termination is the final stage where two free radicals join together or a radical transfers a hydrogen atom to another radical forming a stable product [2-3].

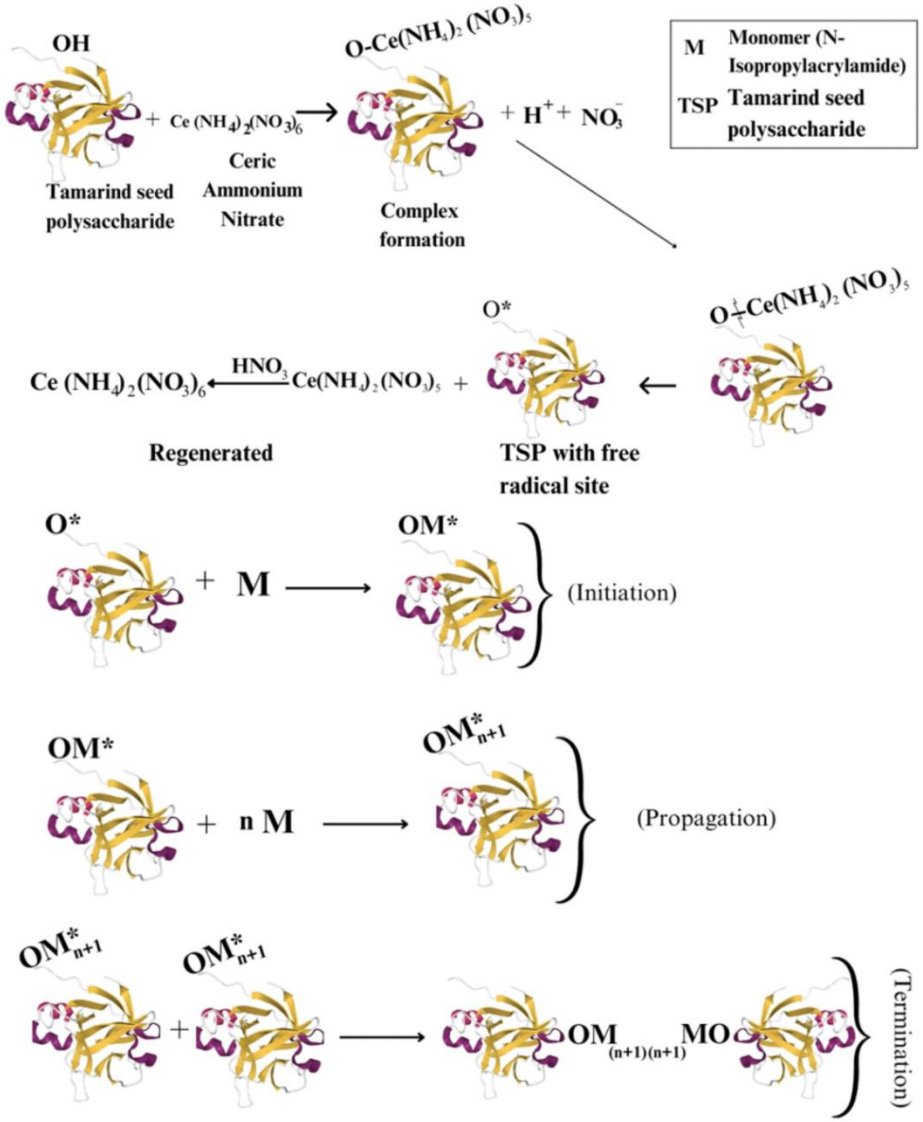

Fig.4.1 Mechanism of grafting of NIPAAm onto TSP

The formation of grafted products was confirmed through FTIR and NMR. FTIR spectra of native TSP, NIPAAm, and NIPAAm-g-TSP are presented in Fig 4.2. The peaks of TSP at 3273 cm^{-1} ascribe to the O-H vibration and at 2926 cm^{-1} for aliphatic -C-H stretching (Fig.4.2A). The peaks at 1647cm^{-1} for -CH-OH stretching vibration, at 1049 cm^{-1} for HC=O stretching vibration, and 756 cm^{-1} for aromatic -C-H group were observed (Fig. 4.2A). A similar peaks of TSP were also reported in the literature [4]. The spectrum of NIPAAm displays an absorption band at 3314 cm^{-1} is ascribed to the N-H stretching vibrations of the primary amide. The peak at 2970 cm^{-1} for asymmetric stretching vibration of $-CH_2$. The two peaks at 1674 cm^{-1} and 1544 cm^{-1} are due to amide-I and amide-II, respectively. The peaks at 2872 and 1386 cm^{-1} are for the stretching vibrations –CH and isopropyl group (Fig 4.2B). These spectra are consistent with those presented in the literature [5]. In the case of graft copolymer a broad band of 3290 cm^{-1} was observed in the FTIR spectrum due to the overlapping of OH-stretching band of TSP and N-H stretching band of NIPAAm (Fig.4.2C). The peak observed at 2970 cm^{-1} for aliphatic -C-H stretching of TSP. The peaks at 2877 and 1367 cm^{-1} are for the stretching vibrations –CH and isopropyl group of NIPAAm. The peaks at 1641 cm^{-1} and 1548 cm^{-1} are due to amide-I and amide-II of NIPAAm. The presence of peaks of NIPAAm and TSP and overlapping of peak indicates grafting.

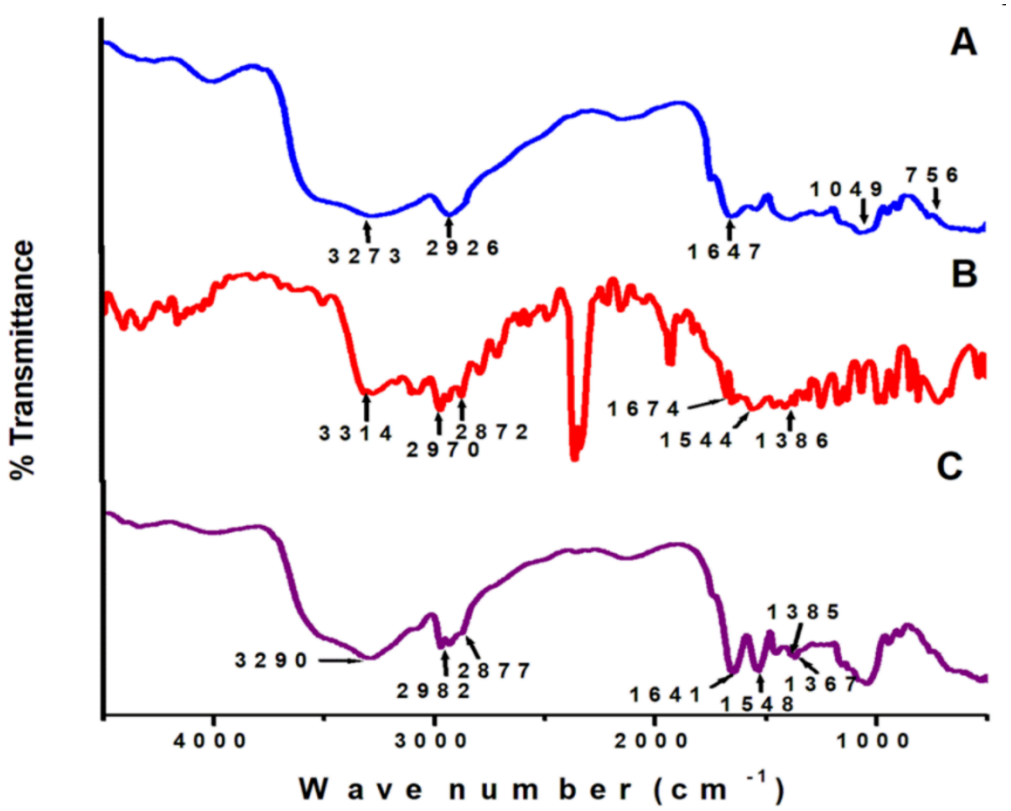

Fig.4.2 FTIR spectra of A) TSP B)NIPAAm C)NIPAAm-g-TSP

13C solid state NMR spectra of TSP and NIPAAm-g-TSP are represented in Fig.4.3 The signal at δ = 105.62 ppm observed in the spectra of TSG is assigned to an anomeric carbon atom [6]. The signals observed at δ = 83.91 ppm for carbon atoms (six-membered ring excluding anomeric one) connected to a hydroxyl group(-OH), at δ = 73.05 ppm for -C-O-C- group in the carbon atom of glycosidic linkage of sugar moieties, at δ = 62.79 ppm for galactose having- CH_2OH group (Fig.4 A) [7]. Three new signals have been observed for graft copolymer. The signals at δ = 181.14 ppm for amide carbonyl carbon atom of -$CONH_2$ groups [8], at δ = 47.74 ppm for (-NCH_2) group resulted from grafting of NIPAAm onto the main polymeric backbone of TSP [9-11], and at δ = 28.93 ppm for vinyl carbon atom of NIPAAm. The above observation confirms the grafting of NIPAAm onto TSP.

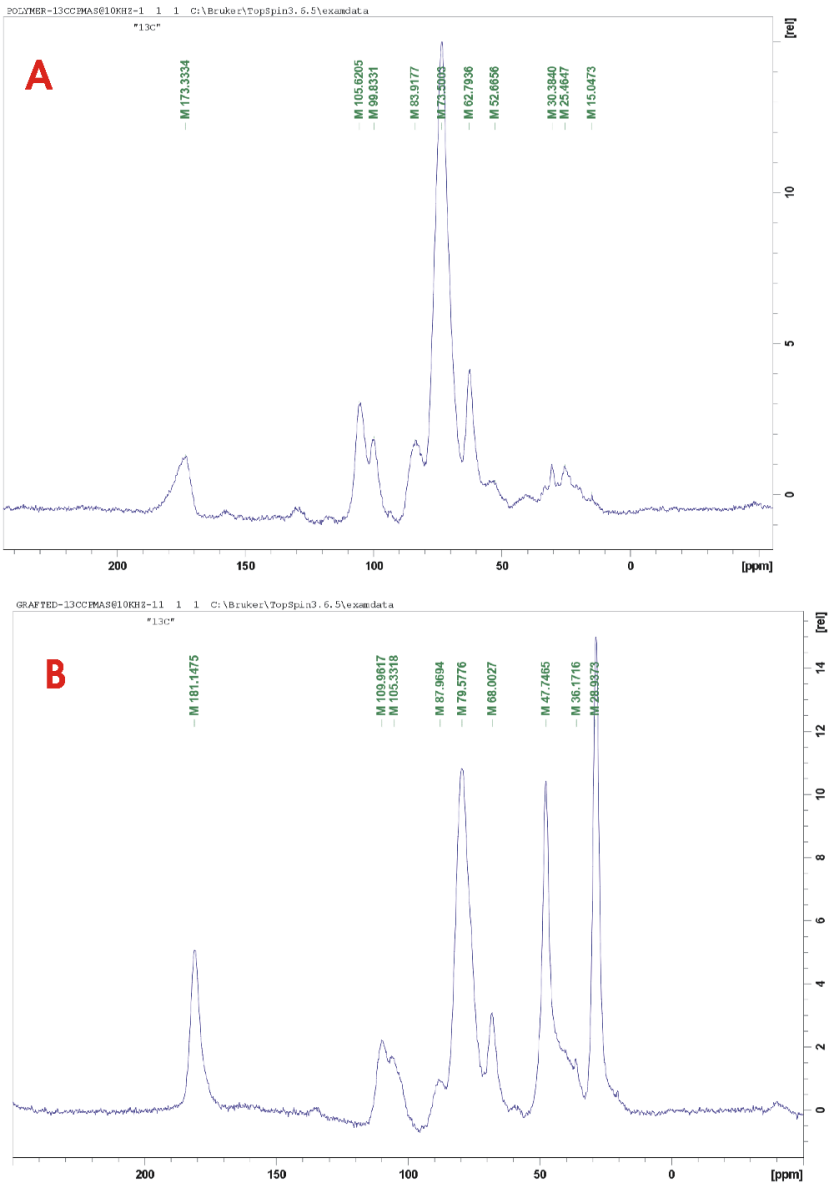

Fig.4.3 Solid state 13C NMR spectrum of (a) TSP and (b) grafted NIPAAm-g-TSP

The grafting parameters, such as the amount of monomer, polymer, nitric acid concentration, and initiator concentration, were varied to optimize the grafting reaction conditions.

The CAN concentration varies from 0.01 to 0.06 (M) (Table 4.1 and Fig.4.4). The grafting parameter increases with an increase in the initiator concentration up to 0.04M. However, a further increase in the initiator concentration may result in a decrease in %G and %GE. The increasing trend may be due to CAN attacking the TSP backbone directly and creating grafting sites that initiate grafting. At higher concentrations, the initiator may cause a reduction in grafting due to an increase in the number of TSP radicals terminated before monomer addition. A similar result was observed by other authors [12]. Therefore, the optimum initiator concentration is 0.04 M.

Table 4.1 % GE and % G at different CAN concentrations

Code	CAN concentration(M)	%G 1	%G 2	%G 3	Mean± S.D.	% GE 1	% GE 2	% GE 3	Mean ± S.D.
	0	0	0	0	*0±0*	0	0	0	0
F1	0.01	0	0	0.24	0.07±0.13	0	0	0.175	0.05±0.10
F2	0.02	1.67	2.33	1.1	1.7±0.61	1.25	1.75	0.825	1.27±0.46
F3	0.03	8.34	9	7.67	8.33±0.66	6.25	6.75	5.75	6.25±0.5
F4	0.04	11.33	12.34	10.34	11.33±1	8.5	9.25	7.75	8.5±0.75
F5	0.05	6.66	6.34	7.34	6.77±0.50	5	4.75	5.5	5.08±0.38
F6	0.06	3.34	4.34	2.67	*3.44±0.83*	*2.5*	*3.25*	2	2.58±0.62

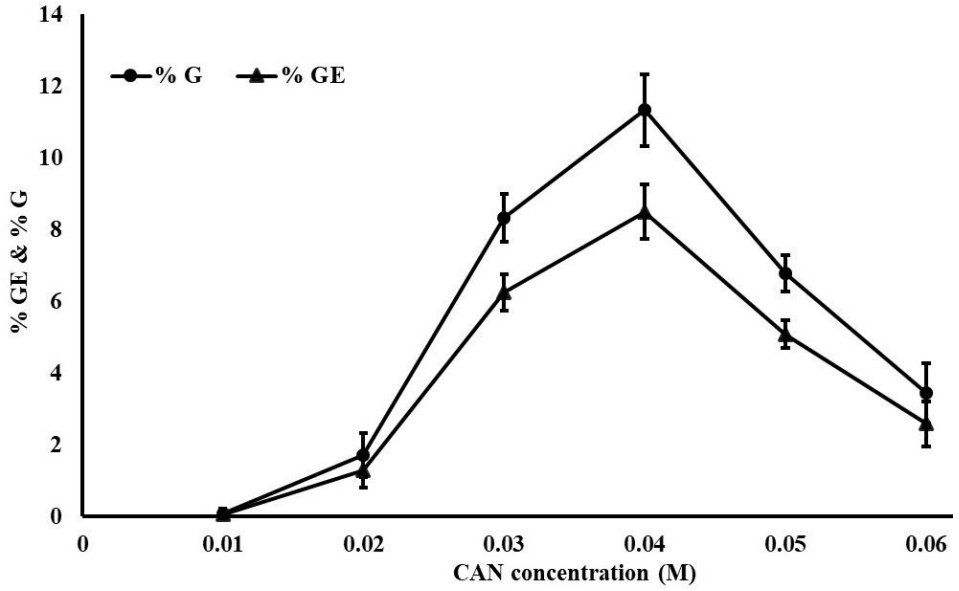

Fig.4.4 % GE and % G at different CAN concentrations

Nitric acid concentrations were changed from 0.1 to 0.4 (M) while maintaining all other reagent concentrations constant. The % G and % GE increase with acid concentration up to 0.2 (M) and decrease beyond this concentration (Table 4.2 and Fig.4.5) The ideal concentration of nitric acid was 0.2 (M). The role of nitric acid in NIPAAm grafting onto TSP is explained by the fact that ceric ions in water are thought to react in the following way:

$Ce^{4+} + H_2O \leftrightarrow [CeOH]^{3+}H^{+}$ ……(III)

$2[CeOH]^{3+} \leftrightarrow [Ce-O-Ce]^{6+} + H_2O$……..(IV)

Initially, the % G and % GE rise because Ce^{4+} ions are converted into H^{+} ions and $[CeOH]^{3+}$ ions. $[CeOH]^{3+}$ is smaller in size and can easily form complexes with TSP. However, at higher concentrations, $[CeOH]^{3+}$ transforms into $[Ce\text{-}O\text{-}Ce]^{6+}$. Due to the larger size of $[Ce\text{-}O\text{-}Ce]^{6+}$, the efficiency of TSP complex formation decreases, resulting in a decrease in % G and % GE. A similar result was observed in graft copolymerization of vinyl monomers onto guar gum by the use of ceric ammonium nitrate as a redox initiator [13]. Therefore, the optimum nitric acid concentration is 0.2 (M).

Table 4.2 %G and %GE at different nitric acid concentrations

Code	HNO3 Concentration (M)	Quantity (µL)	%G 1	%G 2	%G 3	Mean ± S.D.	% GE 1	% GE 2	% GE 3	Mean ± S.D.
F7	0.1	50	11.34	13.33	9.33	11.33±2	8.25	10	7	8.41±1.50
F8	0.2	100	26.67	25.33	28	26.66±1.33	19.75	18.75	21.5	20±1.39
F9	0.3	150	6.67	6	7.33	6.66±0.66	5	3.75	6.25	5±1.25
F10	0.4	200	4.33	6	4.67	5±0.88	3.25	4.5	3.5	3.75±0.66

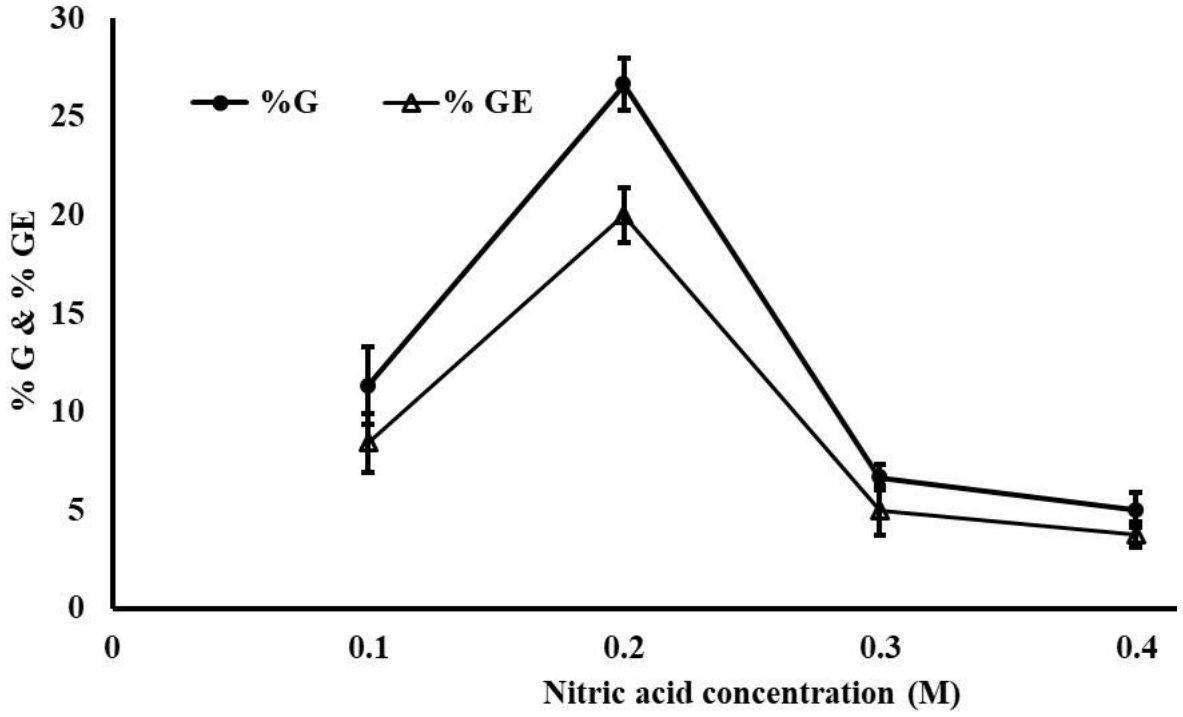

Fig.4.5 % G and % GE at different nitric acid concentrations

The impact of TSP concentration on grafting parameters was investigated by altering TSP amounts from 200 to 350 mg (Table 4.3 and Fig.4.6). Grafting parameters initially increase with higher TSP concentrations but decline beyond 250 mg. The initial increase in grafting parameters may be attributed to the surface activity and self-emulsifying properties of TSP and graft polymer. These properties increase the availability of monomer molecules to TSP's expanding chains and active sites. The declining trend could be attributed to the medium's increased viscosity, which limited monomer access to active regions of the increasing polymeric chain. A similar observation was recorded earlier in the ceric-induced grafting of ethyl-acrylate onto sodium alginate [14]. The optimal TSP

concentration is 250 mg.

Table 4.3 %G and %GE at different polymeric concentrations

Code	Polymer (mg)	%G 1	%G 2	%G 3	Mean ± S.D.	%GE 1	%GE 2	%GE 3	Mean ± S.D.
F11	200 mg	16	16.5	18.5	17±1.322	8	8.25	9.25	8.5±0.66
F12	250 mg	29.6	30	29.2	29.6±0.4	18.5	18.75	18.25	18.5±0.25
F13	300 mg	26	27.33	26.67	26.66±0.66	19.5	20.5	20	20±0.5
F14	350 mg	7.14	7.71	5.71	6.85±1.03	6.25	6.75	5	6±0.90

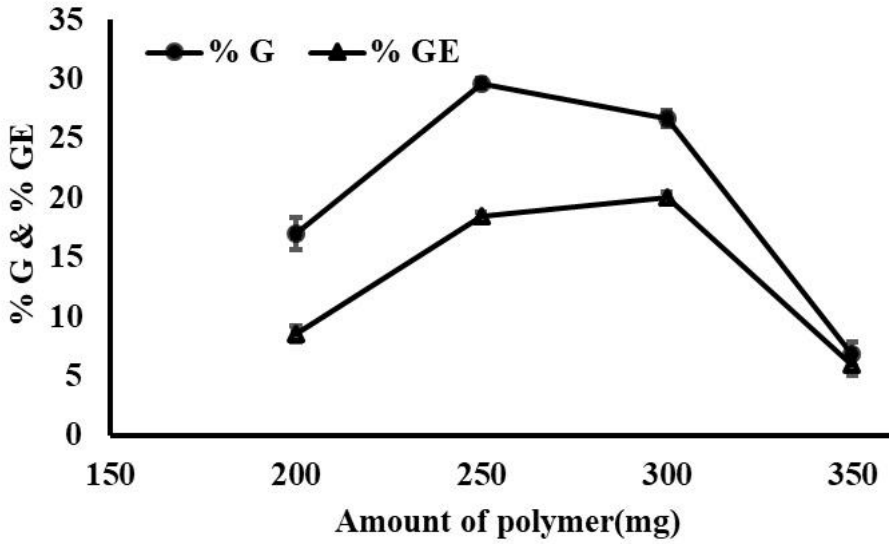

Fig.4.6 % G and % GE at different polymeric concentrations

The monomer content was varied between 350 mg and 500 mg. The grafting parameter increases as the monomer content is increased up to 400 mg (Table 4.4 and Fig.4.7). Beyond this point, the grafting parameter begins to decline. The reason for the increase in grafting parameters with greater monomer content is that more grafting sites become available on TSP macroradicals for monomer molecules. However, at higher monomer content, the grafting parameters decrease because the monomer molecules have a higher affinity for their homopolymer than for the macroradicals. A similar observation was reported earlier [15]. Therefore, the optimal monomer content for this study is 400 mg.

Table 4.4 %G and %GE at different monomer concentrations

Code	Monomer (mg)	%G 1	%G 2	%G 3	Mean ± S.D.	% GE 1	% GE 2	% GE 3	Mean ± S.D.
F15	350	12.8	14.8	14.4	14±1.058	9.14	10.57	10.28	10±0.755
F16	400	30.4	28	30.4	29.6±1.385	19	17.5	19	18.5±0.866
F17	450	22.4	24	23.2	23.2±0.799	12.44	13.33	12.88	12.88±0.444
F18	500	12.4	11.2	13.6	12.4±1.2	6.2	5.6	6.8	6.2±0.6

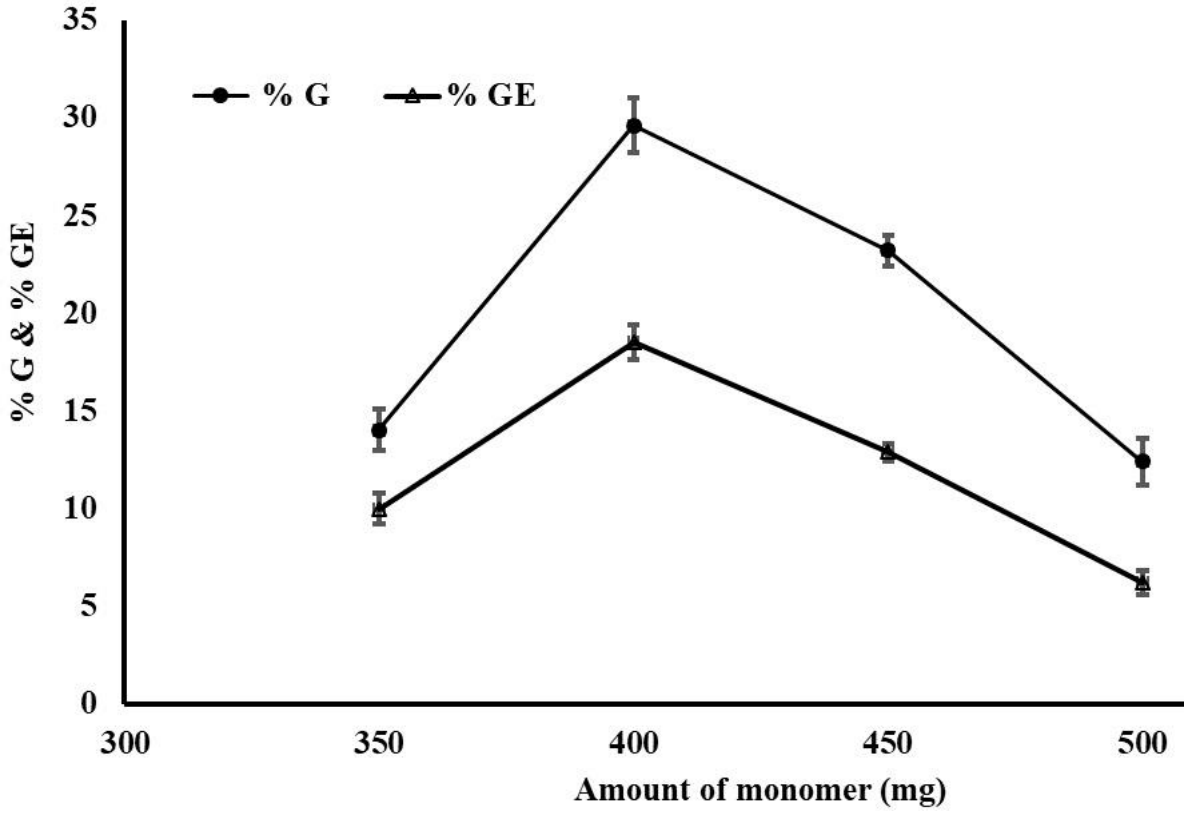

Fig.4.7 % G and % GE at different monomer concentrations

DRZ loaded *in situ* gel formulation was developed using NIPAAm grafted TSP. After thorough visual inspection against a black and white background, all of the created formulations were clear, free-flowing liquids at room temperature that included no particle matter. The pH of all created in situ gel formulations was determined to be in the range of 5-5.5, which is close to the physiological pH of the eye and so would not cause discomfort when implanted into the eye. Tear pH was found to be 7.4 and ranged from 5.2 to 9.3 in disease circumstances. However, the eye may tolerate formulations without buffering in the 3.5-8.5 pH range, and after administration tears correct the physiological pH[16].

When the formulation was put onto the STF at 37ºC, all the formulations gelled within 1-2 sec (Fig.8).

The in situ gel formed remains stable for 1 min to 4 h approximately (Table 4.5). The formulation should have maximum gelling capability, such that after being inserted into the eye as a liquid, it will undergo a quick sol-to-gel transition and keep its integrity without dissolving or eroding for an extended period. It was observed that with an increase in initiator concentration to a certain level, the gelation time decreases, and the gelling period increases, increasing the initiator concentration initially enhances gelling capacity by promoting polymerization and cross-linking, beyond a certain point, it leads to premature chain termination, reduced cross-linking efficiency, and increased heterogeneity. These factors weaken the gel network, ultimately decreasing the gelling capacity. Nitric acid promotes free radical production, which aids in the creation of copolymers and grafting. As a result, a higher concentration of copolymer boosts gelling capacity [17]. In the case of polymers, as the concentration of the polymer grows, it also begins crosslinking, resulting in an increase in the polymer's density, which may generate helix-like structures. However, at lower polymer concentrations, the gelling capacity increases due to the polymer's loose network structure. As a result, creating a gel requires an optimal polymer concentration [18]. Further in case of monomer lower concentration of monomer is needed to form stable gel specially in case of PNIPAM copolymer because the gel network dependent on balance between network connectivity and swelling. So as shown in the table the optimum formulation is found to be F10 which forms gel within 2 sec and remains for 3 h and 20 minute.

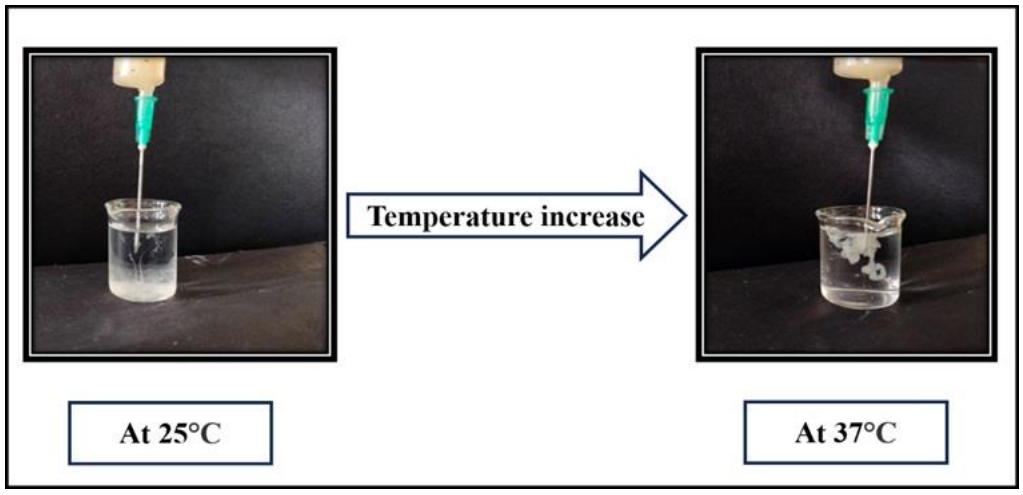

Fig.4.8 Gelling capacity of in-situ gel at 37ºC

The gelation temperature of all the formulations is in the range of 34-38°C (Table 4.5). Ideally, in situ thermo-sensitive ophthalmic formulation is free-flowing liquid at room temperature which turns into gel at physiological eye surface temperature. The gelation at lower or higher physiological temperature will either cause difficulty in instillation or rapid precorneal elimination [19-20]. The formulations F12 synthesized using 0.04 (M) CAN, 0.2 (M) HNO3, 250 mg TSP, and 400 mg NIPAAm exhibited maximum grafting, and gelation at 37˚C were taken to be the optimal formulations.

Table 4.5. Gelling temperature of various formulations

Code	CAN(M)	HNO3(M)	Polymer (mg)	Monomer (mg)	Gelling Temperature (°C)	Gelling Time (Sec)	Gellation Remains(min) (mean±SD,n=3)
F1	0.01	0.1	200	400	38	2	1.06±0.382
F2	0.02	0.1	200	400	38	2	1±0
F3	0.03	0.1	200	400	37	2	1.08±0.127
F4	0.04	0.1	200	400	37	1	8.24±0.816
F5	0.05	0.1	200	400	36	2	7.41±0.776
F6	0.06	0.1	200	400	34	2	5.16±0.505
F7	0.04	0.2	200	400	36	1	9.15±1.165
F8	0.04	0.3	200	400	36	2	8.1±0.765
F9	0.04	0.4	200	400	36	2	7.1±0.83
F10	0.04	0.2	250	400	37	2	200±2
F11	0.04	0.2	300	400	38	2	137±4.358
F12	0.04	0.2	350	400	38	2	77.1±3.637
F13	0.04	0.2	250	350	37	2	94.22±3.287
F14	0.04	0.2	250	450	36	2	70±4.358
F15	0.04	0.2	250	500	34	2	50.02±1.005

The phase transition temperature of the polymer is an important characteristic for the development of an in situ gel. The phase transition temperature of the polymer is caused by a change in its hydrophilic-lipophilic balance [21]. The interactions of polymer with water molecules through hydrogen bonds determine its solubility. The interactions get weaker as the temperature rises. This interaction is strong below LCST but the solution gels above LCST when hydrophobic interactions become more prominent. Thus a decrease in LCST might result from any alteration to a polymer chain structure that increases its overall hydrophobicity.

It was observed that with an increase in temperature, there is a decrease in absorbance and hence a decrease in % transparency (Fig.4.9). This is due to the opacity of the gel, which affects the light scattering. As the light doesn't scatter properly in the opaque solution, the light scattering is decreased, and hence the transparency decreases [22].

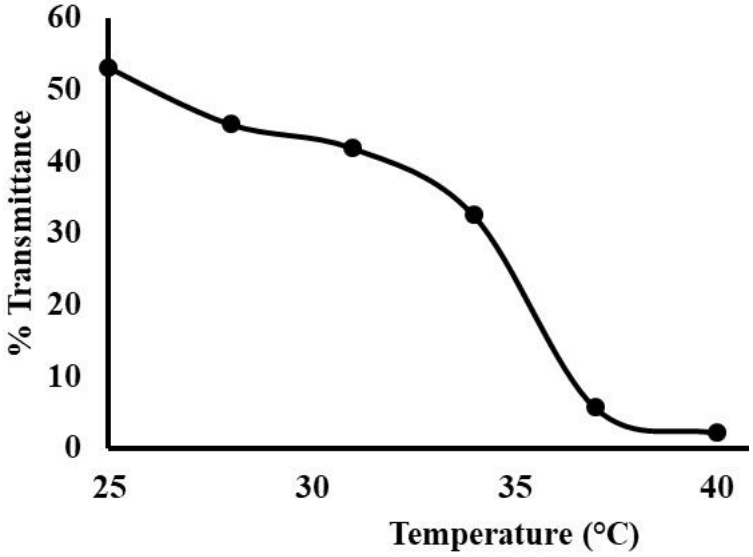

Fig.4.9 Phase transition analysis of the optimized formulation by UV spectrophotometry

The viscosity of the optimized formulation was determined at different temperatures (Fig.4.10). The viscosity was determined at 30 rpm. The formulation was in solution state at 25^{o}C but converted to gel at 37˚C. The viscosity of the formulation rose from 97-372 mPas with an increase in temperature from 25-41˚C. In situ gel formulations should have pre-gelation viscosity of 5-1000 mPas, while post-gelation viscosity should be around 50-50,000 mPas [23-24]. In our case, the solution form's viscosity was 97 mPas and after gelling, it increased to 372 mPas. Thus, the produced formulation demonstrated a range of viscosity that the eye can tolerate well.

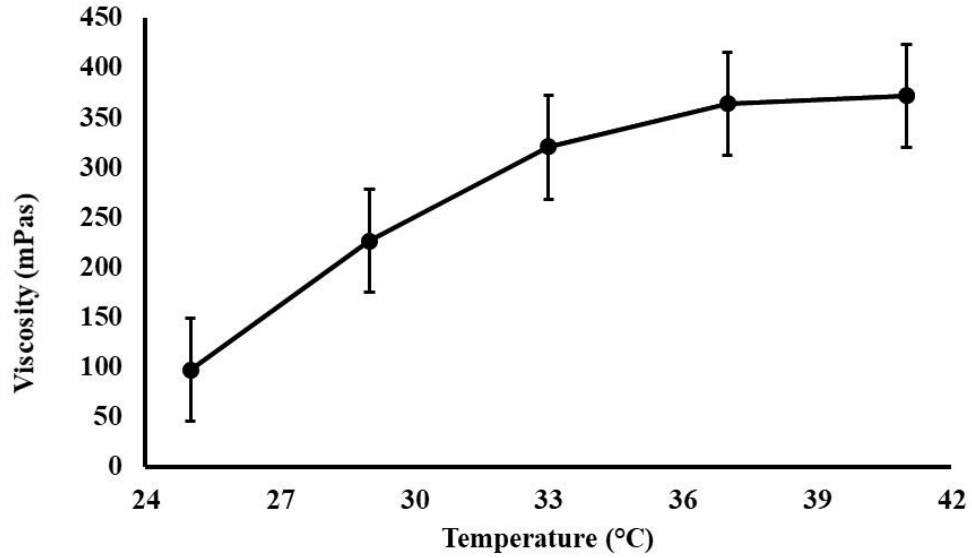

Fig.4.10 Temperature versus viscosity curve

The viscosity of the optimized formulation was studied at various share rates (Fig 4.11). At 25 ^{o}C the formulation was in a liquid state throughout the study and viscosity did not change with increase an in rpm. AT 37 ^{o}C, the in-situ gel formulation showed non-Newtonian flow behavior. The viscosity decreases with increasing shear rate. At rest, the formulation showed high viscosity due to its stable network structure and the strong intermolecular forces. At a high shear rate, viscosity is reduced since the forces applied to the gel are strong enough to break down its internal structure, align its molecules, and reduce internal friction, leading to a lower viscosity. The natural pseudoplastic behavior of the tear film should not disturb through the administration of ophthalmic preparation through the administration of ophthalmic preparation [25]. The in situ gel formulation developed by us exhibited shear thinning behavior meaning high viscosity at low shear stress and low viscosity at high shear rate.

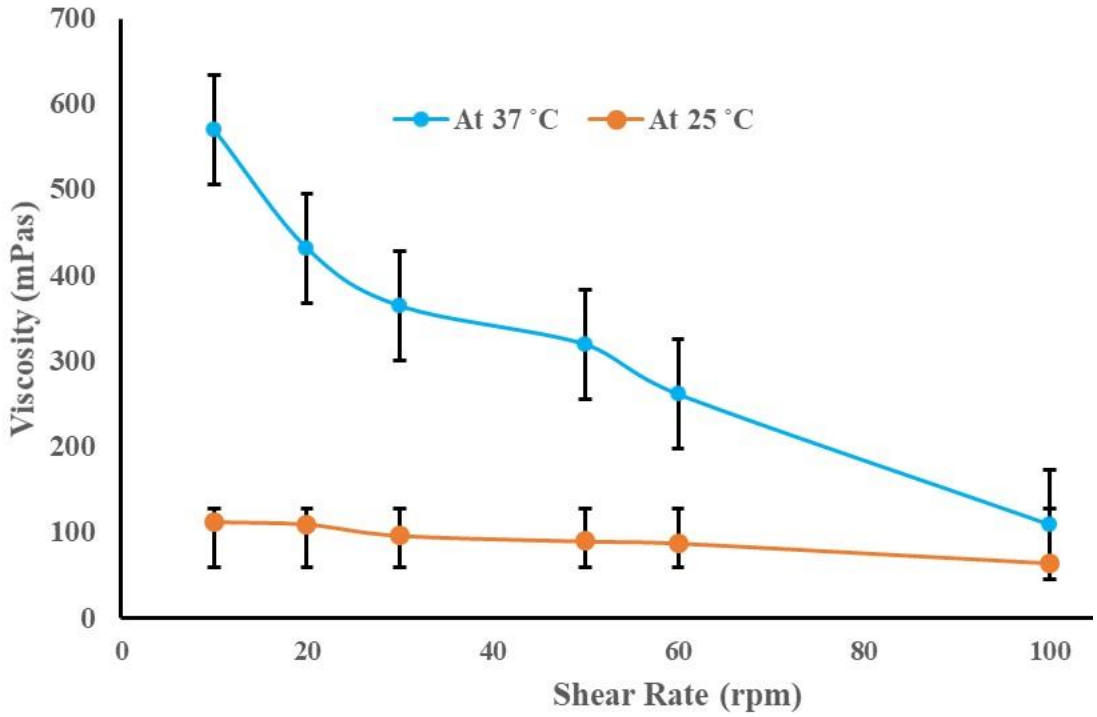

Fig.4.11 RPM Vs viscosity curve

The drug release profile of in situ gel formulation was compared with commercial eye drops (Fig. 4.12). The drug release from developed in situ gel and commercial eye drop after 2h were 33.97 and 66.07%, respectively. The complete drug release was observed within 5h from the marketed formulation. Only 53.84% drug was released from in situ gel formulation within 5h.

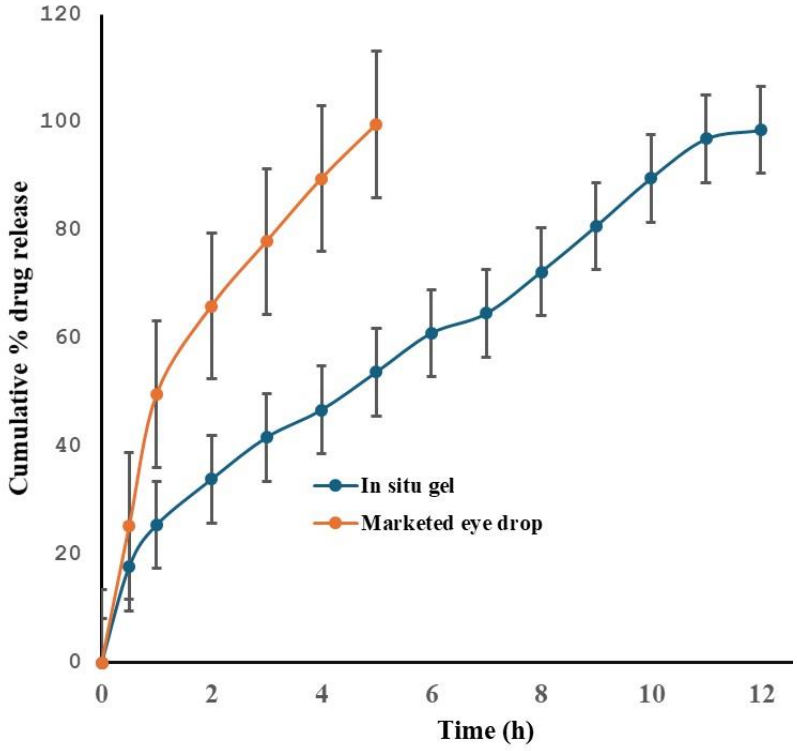

Fig.4.12 In vitro release study

The ocular irritation study of in situ gel formulation was carried out on goat cornea. The microscopic examination of cellular and tissue modifications is represented in Fig.4.13. The negative control and test control depicted that the epithelial cells were intact, healthy, and attached to Bowman's membrane (Fig.4.13 a & Fig. 4.13c). In contrast, the positive control showed the disruption of epithelial cells, and some parts of the epithelium were not properly attached to Bowman's membrane (Fig. 4.13b). It was found that the optimized in situ gel exhibited low toxicity and did not show any disruptive effect on the corneal epithelium or stroma when the cornea was incubated with the optimized formulation. The same result was found in the negative control, whereas the positive control, treated with 0.1%SDS, affected the keratinocytes by decreasing epithelial junction integrity.

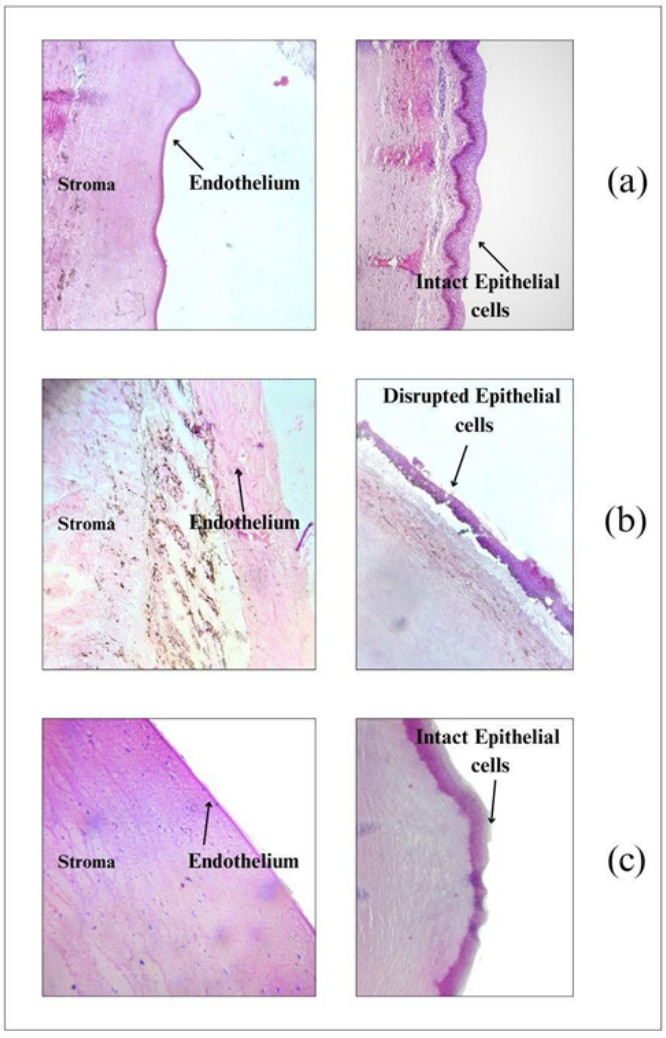

Fig.4.13 Ocular irritation study on goat cornea a) normal saline (negative control) b) 0.1% sodium dodecyl sulfate (SDS) solution (positive control) c) optimized in situ gel formulation

The ex-vivo permeation of the drug through goat cornea was compared between the optimized formulation and the marketed formulation (Fig.4.14). The data depicted that the drug permeated from the optimized formulation was 27.43 % within 6 h, while the drug permeation was about

16.54 % in the marketed formulation. The permeation of the optimized formulation is higher than the marketed one, which may be due to the polymer's mucoadhesive properties, allowing more drugs to permeate into the cornea of the eye and enhancing the bioavailability of the drug [26].

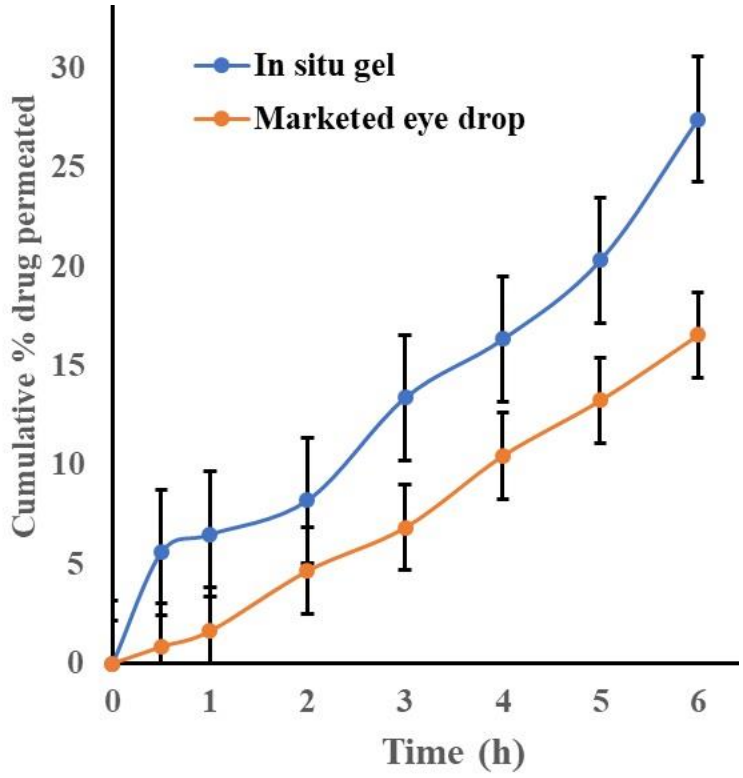

Fig.4.14 Ex vivo permeation study

Isotonicity has to be maintained to increase the drug efficacy and prevent epithelial damage, tissue damage, or any ocular irritation of the eye. The optimized solution containing DRZ was subjected to isotonicity study. It was found that there is no change in the shape or size of the RBC (bulging or shrinking) when compared with the pure RBC shown in Fig.4.15. Hence the formulation was found to be isotonic [27-28].

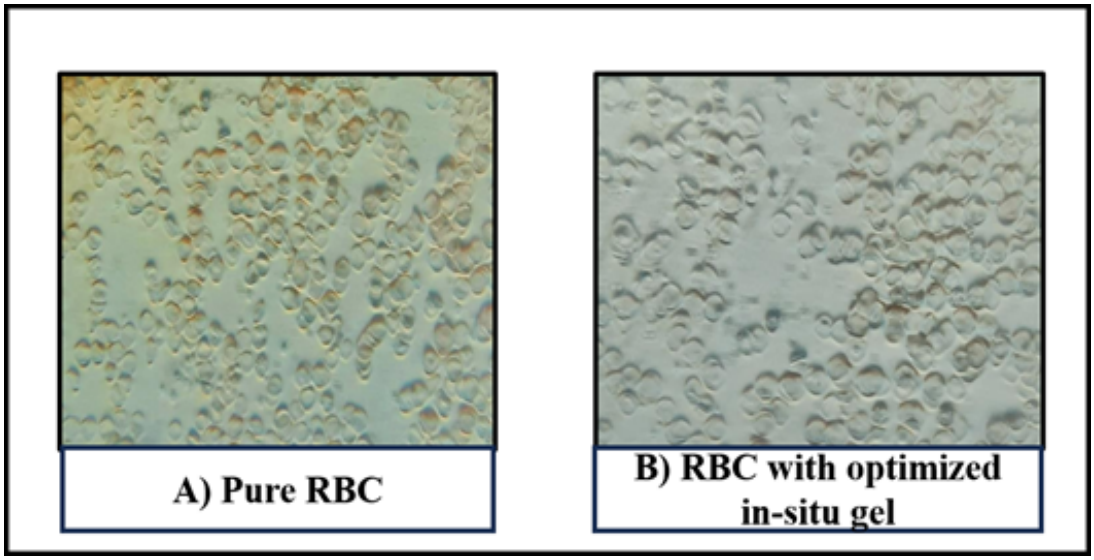

Fig.4.15 Isotonicity study a) Pure RBC b) RBC with optimized in-situ gel

Conclusion

Grafting of TSP with NIPAAm was performed by the free radical graft co-polymerization technique. Non thermo-sensitive TSP converted into thermo-sensitive TSP. FTIR and NMR studies confirmed the grafting of TSP with NIPAAm. DRZ loaded in situ gel forming solution was developed using optimized grafted TSP. The optimized formulation displayed an LCST at 37˚C which is ideal for ocular in situ gels. The *in situ* gel also had an optimum pH and viscosity range for application to the ocular *cul-de-sac*. DRZ release from the *in situ* gel was sustained up to 12 h, outperforming that of the commercially marketed formulation. The developed formulation does not cause any eye irritation. From the rheological study, it was observed that all the formulations exhibited pseudoplastic flow as evidenced by a decrease in viscosity with an increase in angular velocity. The permeation of the drug is enhanced as compared with the marketed one. The study indicated that *in situ* gel formulations are promising candidates for the treatment of various ocular diseases. We are hopeful that our research will guide future studies on ocular *in situ* gel for the treatment of dry eye syndrome, age-related macular degeneration, and other diseases of the posterior eye.

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