Tacrolimus loaded lipid-polymer hybrid nanoparticles incorporated thermosensitive gel as intravesical drug delivery system

Abstract

A drug that is administered through a catheter into the bladder is referred to as an intravesical drug delivery system. The aim of this work is to improvise intravesical drug delivery of Tacrolimus loaded lipid polymer hybrid nanoparticles thermosensitive gel for bladder wall to provide control release, increase intravesical residence time and avoid fast removal by urination. The Tacrolimus loaded lipid polymer hybrid nanoparticles mimic cell membrane and enhance cellular uptakes. Our data reveals nanocarrier formation with a mean particle size, zeta potential and entrapment efficiency of optimal formula was found to be 124±0.01 nm,-27.5±0.102mv and 72% ± 0.15% respectively. In situ gelling formulations containing TAC-CS-LPHNs can adhere to the mucosal layer of the bladder and help in the diffusion of therapeutic agent across the bladder wall. To impart mechanical strength and mucoadhesive properties, the chitosan concentration was adjusted to 0.5% and 0.25%.The image of field emission scanning electron microscope shows an irregular surface with filled pores with nanoparticles. The gel was syringeable and had reasonable viscosity within room temperature. A Tissue uptake study reveals that Tacrolimus loaded lipid polymer hybrid nanoparticles have been penetrating bladder tissues and accumulated inside the cytoplasm and nucleus of urothelium epithelial cells. In addition, the bioimaging study shows that the gel resided inside the bladder for up to 2 hrs and completely urinated out of the bladder with negligible distribution to other tissues.

Keyword: Tacrolimus, Intravesical drug delivery, lipid polymer hybrid.

Introduction

The effectiveness of drugs for local bladder therapy depends on its ability to pass through urothelium and physicochemical properties of the drug itself, like the MWT (less than 200 Da), lipid solubility and partition coefficients (1).

Typically, approximately 50 mL of drug formulation may be instilled intravesically (2), and micturition is avoided for at least 1-2 hours to ensure successful drug transport into the underlying tissues (3). Nevertheless, due to the presence of residual urine, drug formulation will be diluted and wash out frequently (4). Due to these limitations, intravesical drug delivery can result in repeated catheterization, frequent dosing, also may lead to urinary tract infection and irritate the urinary endothelium (5).

Advanced drug carriers have been designed to enhance the solubility of hydrophobic drugs, the diffusion of hydrophilic drugs through the urothelium, the adhesion of drug carriers to the urothelium, and the uptake/permeation of drugs into bladder tissues over a prolonged period of time. An example of these carriers are a Particulate incorporated hydrogel (7), modified nanoparticles, and solubilized amphiphile (6).

Hydrogel systems can act as drug depots, increasing the residence time of drug in the bladder, due to their bio-adhesion to the bladder mucosa (8). A hydrogel that is particularly appealing is one composed of injectable thermosensitive polymers that form gels spontaneously at the target site. Due to the fact that the body temperature is about 37°C, thermosensitive gelation of the instilled polymeric dispersion is likely to occur inside the bladder. Once created inside the bladder, the hydrogel can act as a matrix for drug delivery (9). Poloxamer 407 is a thermosensitive polymer. It is soluble in water up to the lower critical solution temperature (LCST), but transforms into a hydrogel above this point. Poloxamer 407 can be coupled with natural polymers such as chitosan to enhance its mechanical strength and mucoadhesive properties (10).

Bladder pain syndrome/Interstitial cystitis (BPS/IC) is classified as a chronic inflammatory bladder condition, hallmarked by the presence of chronic pain. Interstitial cystitis is a life modifying disease characterized by chronic pelvic and bladder pain, frequent urination, and suprapubic pain (11). The main corner stone in the pathology of BPS/IC is the destruction of the glycosaminoglycan (GAG) layer (12).

Intravesical administration will help in providing high drug concentration at the site of action and eliminate the side effects associated with oral or parenteral therapy, in addition to a small quantity of drug that fractionated from the kidney to reaching the bladder (12).

Chuang et al. study the effect of liposomal encapsulated tacrolimus on the bladder of cyclophosphamide induced cystitis. He proposes that Tacrolimus inhibited substantially the inflammatory cystitis of cyclophosphamide by modifying the activity of IL2, PGE2 and EP4. These findings support local tacrolimus research of inflammatory cystitis refractory to conventional treatment (13).

The invention of such novel system which combines liposome and polymeric nanoparticles in one nanoparticle results in major improvement in drug delivery systems (3).It is composed of three distinct components, the polymeric core which is mostly hydrophobic in nature, where the drug is entrapped inside and the outer shell that is lipids in nature and help in retarding the drug inside and impart mechanical strength and control release (4). The outer most layer is the lipid-PEG layer that surround the lipid shell help in stabilization of the nanoparticles by decrease the aggregation through a steric hindrance also prolong in vivo circulation of CS-LPHNs (5,6).

Experimental

Materials

Tacrolimus monohydrate was purchase from Hangzhou Hyper Chemicals Limited (China).1,2-distearoyl-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene-glycol)-2000](DSPE-PEG2000) and Soya bean phosphatidylcholine were purchase from Avanti Polar Lipids, USA. Poly (D, L-lactide-co-glycolide) lactide:glycolide (PLGA) (75:25), mol wt 66,000-107,000 was purchased from Sigma-Aldrich, Chemie GMBH, Germany, Poloxamer 407 purchase from Prill, BASF, USA and Chitosan LMW (MW 100000 Da; DD 98%) purchased from Giusto Faravelli, Milan, Italy. All the other chemicals were of analytical quality.

Preparation of Tacrolimus monohydrate loaded lipid polymer hybrid nanoparticles (TAC-CS-LPHNs)

The preparation of TAC-CS-LPHNs and characterization were described thoroughly in our previous article (26). Briefly, a self-assembly single step nanoprecipitation method has been used. Organic phase containing TAC and PLGA was dropped using insulin syringe onto aqueous phase which dissolving lecithin and DSPE-PEG_{2000} under magnetic stirring. The resulting solution was sonicated for 3 min at frequency of 42 kHz and power of 50 W. The unassembled component and the acetonitrile were removed by washing TAC-CS-LPHNs solution three times using an Amicon® Ultra-4 centrifugal filter unite (MWT 10000 Dalton) at rate 1000 rpm which may resuspend again in deionized water to obtain desired concentration. The resulting TAC-CS-LPHNs were stored at 4˚C, or freeze dried and lyophilized for storage at -20˚C (14).

Preparation of TAC-CS-LPHNs thermosensitive mucoadhesive in situ gel

Using poloxamer 407 and low molecular weight chitosan, a gel was prepared using the cold technique. A calculated amount of TAC-CS-LPHNs (each 7.2 mg equivalent to 1mg TAC) was prepared as a colloidal dispersed solution. Previous weighed amount of poloxamer 407 was added for cold solution of TAC-CS-LPHNs (5-10 ˚C) over 2-3 min under magnetic stirring as in table (1). Fast adding of the powder to water should be avoided because it will lead to formation of large ball which required many hours to dissolve. A low MWT chitosan was dissolved in 0.1 M HCl solution under magnetic stirring. Both solutions were mixed in different ratio under stirring for 15 min to obtain mixtures containing poloxamer and chitosan as in the table (1). The pH was adjusted to 6.5. The fluorescently labeled Gel was prepared by using FITC-TAC-CS-LPHNs instead of TAC-CS-LPHNs. Also, 6-Coumarin (6-C) loaded in situ gel was prepared by using 6-C-CS-LPHNs instead of TAC-CS-LPHNs.

Table (1). The formulas of TAC-CS-LPHNs in situ gel

Formula Code	TAC-CS-LPHNs(mg)	Poloxamer 407(w\w%)	Chitosan (w\w%)	Deionized water(g)
PX-1	28.8	20	0.25	100
PX-2	28.8	20	0.5	100
PX-3	28.8	18	0.5	100
PX-4	28.8	18	0.25	100

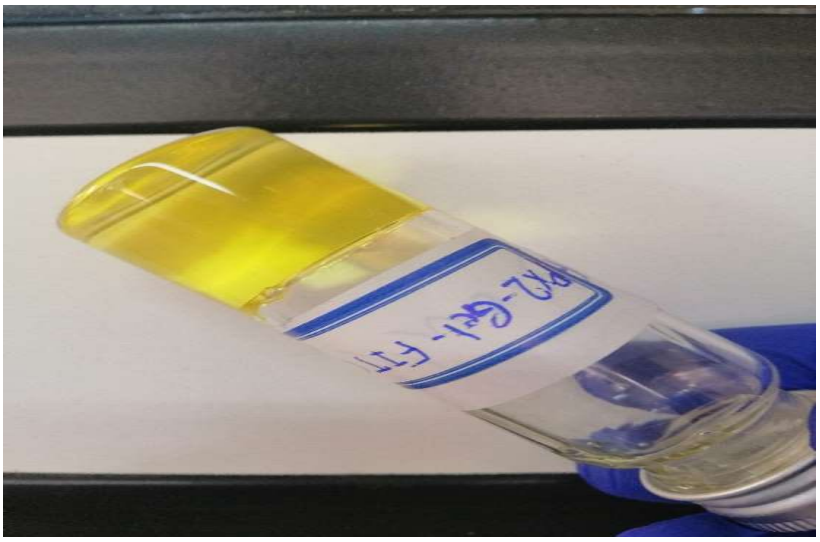


Figure (1). FITC-TAC-CS-LPHNs in situ gel

Characterization of TAC-CS-LPHNs in situ gel

Field Emission Scanning Electron Microscope (FESEM) Analysis

The FESEM was performed to get a better understanding of TAC-CS-LPHNs in situ gel morphology and confirm the particle size obtained from DLS using Field Emission Scanning Electron Microscope (FE-SEM), NovaNano SEM-600, Netherlands.

pH measurements

The pH value of the TAC-CS-LPHNs in situ gel was measured by means of pH 210 Microprocessor pH Meter.

Gelation temperature

Sol-gel transitions is an important parameter in evaluating the formulations. 5 mL sample was taken in a glass vial and was placed on a magnetic stirrer (150 rpm) with the heating arrangement. The temperature was gradually increased at the rate of 2˚C/min. The temperature at which the rotation of the magnetic bead was stopped was recorded as the gelation temperature (15,16).

Gelation time

The gelation time required for gel to transform from a solution to gel state was determined at 37˚C. Vial inversion method has been used to determined gelation time in the bladder environment. Briefly, 5 mL of the sample was kept in glass vial and stored in water bath at 37˚C. The vial was inverted at a specified time to test the gel flow, and the time at which the sample fails to flow is described as the gelation time (17).

Viscosity measurement

Viscosity is also one of the parameters to be evaluated for in-situ gels. The viscosity of the formulations should be such that it remains convenient during their administration by the patient. Viscosity of the formulation was determined at different temperature using Brookfield viscometer, Brookfield Engineering, USA (18,19).

Mucoadhesive strength

The mucoadhesive force was determined using modified two-pan balance method. In vitro tests including two-pan balance method is the most common and convenient methods to assess the mucoadhesive properties of formulations. One side of the balance was provided with an appropriate place for the weight, and the other was designated for use as a place for sheep's bladders. Gradually water added drop by drop untill the bladder tissue got detached from the gel (20). The weight of water necessary to separate the two surfaces was calculated, and mucoadhesion was found to be measured in grams.

$$F=G*W$$

Where F is the muco-adhesion force (dynes / cm^2),

W weight (grams),

G is the acceleration due to gravity (cm/s^2).

Syringeability of formulations

Measurement with a syringe was used to determine the force done to extract the formulation was accomplished using a texture analyzer (Stable Micro Systems Ltd, UK) connected to a force sensor. The gel was loaded in syringe that attached by urinary catheter. The syringe pushed with a constant pressure at force of 0.5N. The resistance to syringe contents being expressed through the catheter during plunger compression at 22˚C (17).

Dilution of TAC-CS-LPHNs in situ gel

Due to the presence of urine in the bladder, dilution of the gel will happen and may affect characteristics of the gel. 50 mL of in situ gel solution was diluted with an equal amount with 1:1 ratio of artificial urine and evaluated for changes in gelling properties.

Animal studies and in vivo bioimaging

Animal welfare statement

All animal procedures performed in accordance with ethical protocol approved by University of Baghdad /Research Ethics Committee/ethical code ***RECAUBCP112019*** and conducted in Tehran university for medical science/preclinical core facility (TPCF).

Bladder Instillation.

Female mice were anesthetized with 100 mg/kg and 10 mg/kg of ketamine and xylazine respectively, then catheterized with a lubricated catheter of 24G cannula. The bladders were emptied manually and then irrigated with sterile PBS followed by the installation of 50µL of solution (6-C-CS-LPHNs in situ gel). An ultra-small clamp was placed on the external urethra for two hours to prevent the bladder from expelling its contents (21).

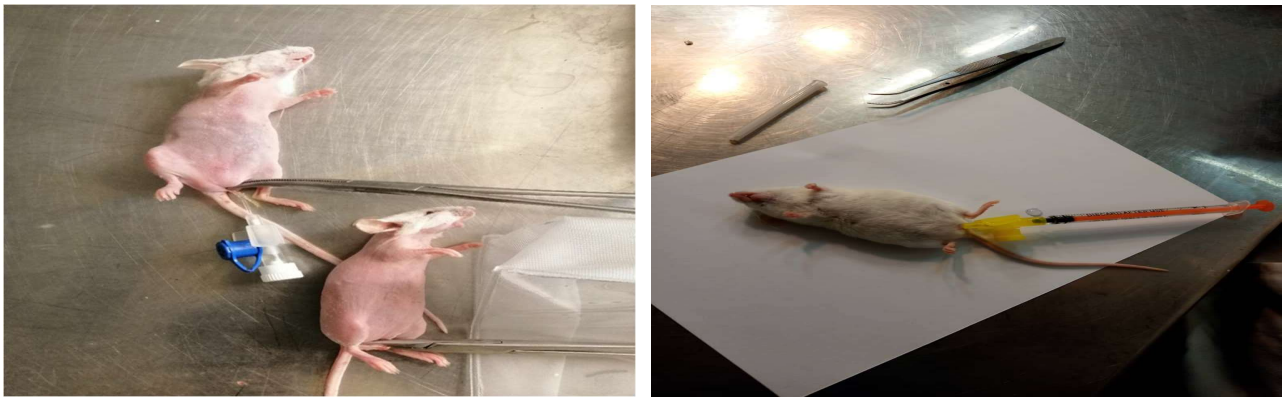


Figure (2). Bladder installation for mice

6-C-CS-LPHNs uptake and retention by bladders wall

Three female mice weighing 200 g was supplied by the animal house. The solution then installed into bladder as describe previously. One mouse was injected with 50 µL of PBS as a control group and the other was injected with 50 µL of 6-C-CS-LPHNs after 2 hrs, the remaining TAC-CS-LPHNs should washed extensively with PBS before mice scarified and bladder extracted. The bladder wall was cut into sections of 200 µm sections (thickness) and each of these sections was applied to glass slides, where it was fixated using a wax and imaged by confocal laser scanning microscope (CLSM) at 20X magnification (22).

Intravesical retention time and bioimaging

Twelve mice were shaved well before injected intravesically because hair may have fluorescence emission and interfere with bioimaging study, anesthetized and kept in room temperature.

The mice were classified into four groups and bladder injected as the following

A. Three mice was given PBS consider as the control group and given code (I)
B. Six mice were injected with FITC-CS-LPHNs solution and given code (II) and (III)
C. Three mice was injected with FITC-CS-LPHNs in situ gel and given code (IV)

The mice were visualized by kodak in vivo imaging system fx pro, KODAK, USA on predetermined time (1,2,3,4,5,6 and 24hrs.) at 460 and 520 nm for excitation and emission, respectively. Fluorescence pictures were taken using Living Image 4.5.5.

Figure (3). Preparation and bladder installation of the mouse with in vivo imaging.

Statistical studies

All experiments were repeated three times, and data are presented as mean±S.D. Statistical significance was calculated using a two-way ANOVA using Minitab program by setting the significant as level as $p<0.05$.

Results and discussion

Intravesical drug delivery is a term that refers to drugs that are administered via catheter into the bladder. This local route of administration of medication has been investigated for the treatment of a variety of bladder disorders, including bladder cancer, infection, and bladder pain syndrome (23).

The goal of this article is to improvise intravesical drug delivery of TAC-CS-LPHNs for bladder wall to provide control release, increase intravesical residence time and avoid fast removal by urination.

The TAC-CS-LPHNs was prepared and characterized. The mean particle size, zeta potential and entrapment efficiency of optimal formula was found and discussed in separated article (26).

Certain polymers, such as poloxamer and chitosan, having thermosensitive and mucoadhesive in situ gelling properties. Chitosan has the ability to form non-covalent bonds with glycoprotein components of bladder mucin through electrostatic, hydrogen bond and chain entanglement (24). They are preferred for design of intravesical drug delivery system because can overcome some limitation of this system like minimize bladder washout and increasing residence time in bladder.

The TAC-CS-LPHNs-loaded poloxamer\chitosan in situ gel, can create a bioadhesive gel layer over a large surface area of the urothelium, enabling high concentrations of the drug to diffuse for a prolonged period of time across urothelial tissues. The common method used in laboratory to prepare thermosensitive in situ gel refer as cold method. Water was cooled for less than 10˚C and the poloxamer was added slowly over 20 mins under gentle stirring to help in hydration of the flakes surface with increase rate of dissolution and prevent aggregation which may require several hours to dissolved again. Due to weak mechanical properties and rapid erosion of poloxamer 407, we added chitosan. The major limitation in use of chitosan in a concentration more than 2% is increasing viscosity of resulting gel (25). Therefore, we use 0.5% and 0.25%, to impart mechanical strength and mucoadhesive properties.

The pH of the gel was adjusted to approximately 6.2, so it doesn’t induce irritation in the bladder when administered intravesically using 0.1M NaOH.

Field emission scanning electron microscopy

Surface morphology of Intravesical gel was visualized using (FESEM). The image of gel shows irregular surface with filled pores with nanoparticles of drug which favored entry of water and release of TAC-CS-LPHNs.

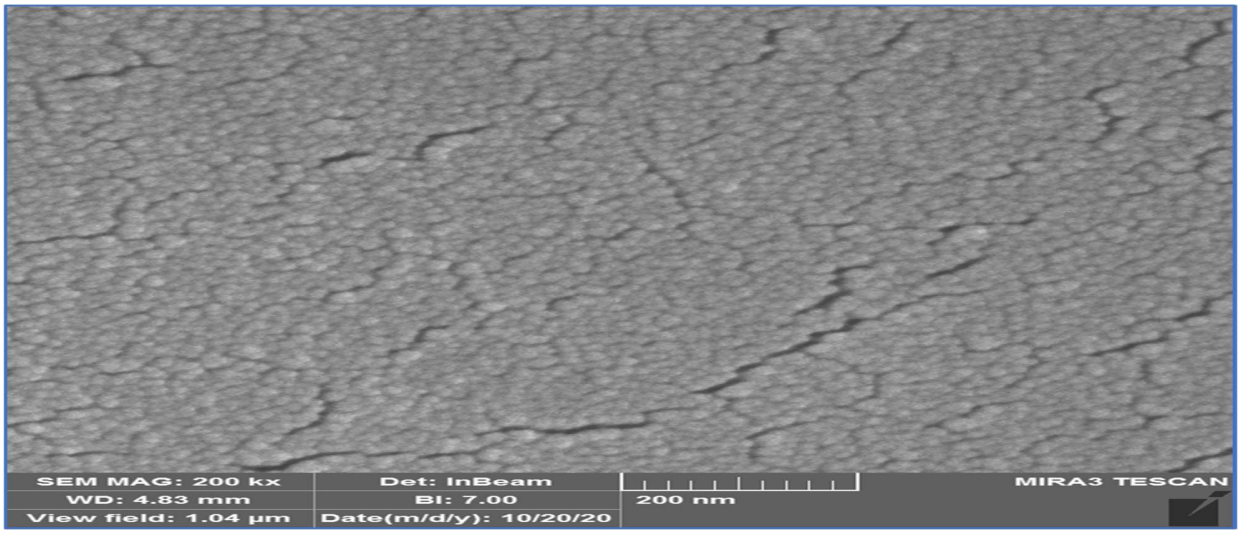


Figure (4). FESEM image of Mucoadhesive Thermosensitive in situ Gel.

Viscosity measurements

The viscosity of the gel may affect gel properties like syringeability when injected through catheter and spreadability in the bladder, for this reasons, viscosity of the prepared formulation was evaluated at different temperature using Brookfield viscometer at different RPM and spindle number in order to adjust the viscometer at higher desirability percentage. The result was showing in figure (5), the figure reveals an increase in viscosity of the gel with an elevation in temperature. Increase in the temperature will decrease in the viscosity of chitosan solution due to increase in thermal kinetic motion of chitosan polymer. On the other hand, increase in the temperature lead to marked increase in viscosity of poloxamer 407 solution due to thermo-gelling properties of poloxamer (25). From these observations, we can conclude that chitosan has a negative effect on the viscosity of poloxamer-chitosan gel at increasing temperature. Also, an increase in concentration of poloxamer 407 lead to increase the viscosity of the gel. This result is desirable for bladder formulation as the temperature of the body will increase viscosity of the gel.

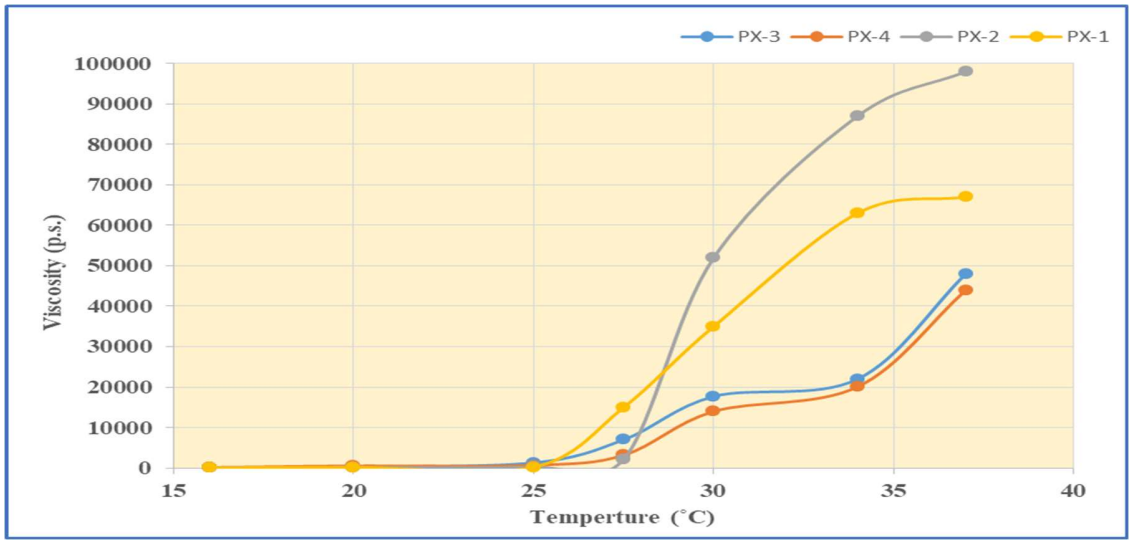


Figure (5). The viscosity of formulated gels at different Temperatures.

Syringeability

Syringeability is a valuable test for evaluating intravesical dosage form. Since the administration of intravesical dosage form through the catheter, it is crucial for poloxamer-chitosan in situ gel to pass through the catheter freely and at the temperature (22˚C).

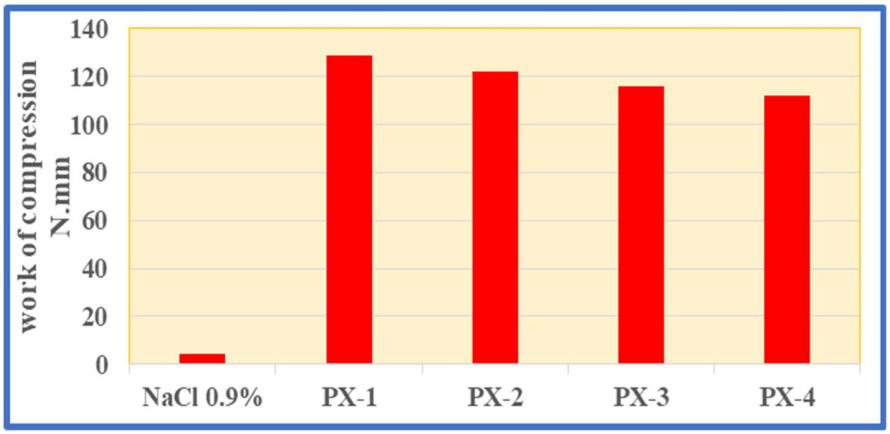


Figure (6). Syringeability of TAC-CS-LPHNs loaded in situ gel.

Figure (6) reveals the work required for injection of in situ gel in the catheter through syringe, that inversely related with syringeability through urinary catheter. Data show significant ($P< 0.05$) increase in the work of compression in compare with NaCl solution. Also, concentration of chitosan shows insignificant changes in work of compression when comparing PX-1with PX-2 while PX-4 shows a significant ($P< 0.05$) decrease in work when compare with PX-1. Increase in the concentration of poloxamer polymer cause significant ($P< 0.05$) increase in the syringeability work force required for injection.

Gelation temperature / Sol–gel transition temperature ($T_{sol-gel}$)

Phase transition temperature ($_{Tsol-gel}$) is the temperature at which the liquid converted into gel and consider as important parameter for in situ gel. The optimal $_{Tsol-gel}$ should be between 25 ˚C (the room temperature and 35˚C close to (body temperature).

The Tsol-gel of different poloxamer-chitosan in situ gel formulas was shown in figure (7). It obvious from the figure that Tsol-gel affected by polymer concentration, as increase in concentration reduce $T_{sol-gel}$, in addition to that chitosan concentration shows little effects on $T_{sol-gel}$.

Because that gelling mechanism of poloxamer is depending on the micelle packing and entanglements, the addition of drug or other polymer can affect micelle formation and may modify Tsol-gel.

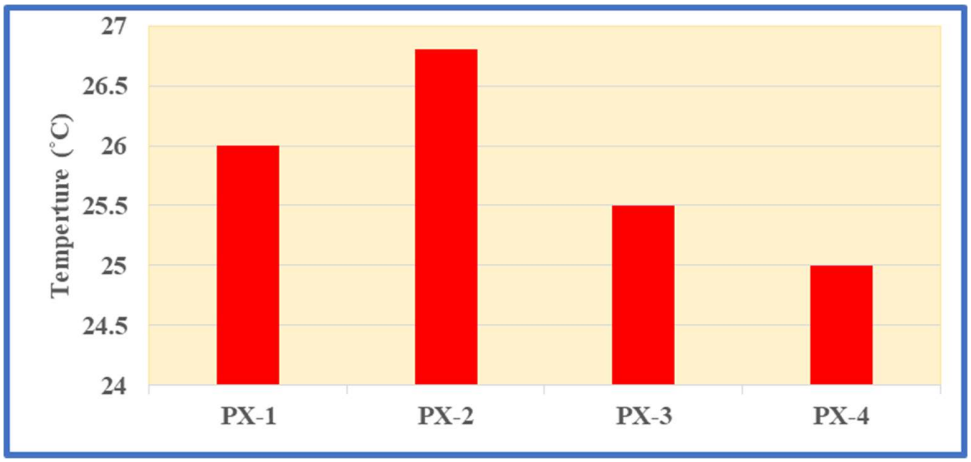


Figure (7). $T_{sol-gel}$ of poloxamer-chitosan in situ gel formulas.

Dilution of TAC-CS-LPHNs in situ gel

Due to incomplete empty of bladder, there is about 50 mL of urine will stay as residual volume in the bladder even after voiding that lead to dilution of in situ gel and change in its properties. Generally, the in situ gel was administered by catheter in a volume of 50-100 mL. Therefore, 50 mL of in situ gel was diluted with 50 mL of artificial urine (1:1) and the changes in properties were studied.

There is a significant ($P<0.05$) loss of viscosity after dilution especially at 37˚C and increase $T_{sol-gel}$ due to decrease in concentration of polymer.

Table (2). The viscosity and gelation temperature of in situ gel after dilution.

Formula	Viscosity (P.s.)				Gelation temperature (˚C)	
	25 ˚C		37 ˚C			
	Before dilution	After dilution	Before dilution	After dilution	Before dilution	After dilution
PX-1	240±21.2	136±12.	67000±23	22000±33	26±0.27	27.3±0.14
PX-2	259±8.12	142±22.	98000±34	36000±34	26.8±0.1	28 ±0.17
PX-3	1310±12	511±26.	48000±28	18000±17	25.5±0.05	26.4±0.05
PX-4	700±23.5	231±10.7	44000±56	12000±37	25±0.19	26.1±0.21

Gelation time

All poloxamer-chitosan formulas show fast and complete gelling with in physiological temperature 37˚C. Figure (8) reveals the time required for gelling.

A significant increase ($P<0.05$) in gelation time was indicated with decrease in poloxamer concentration while a significant increase in gelation temperature was detected with increase chitosan concentration.

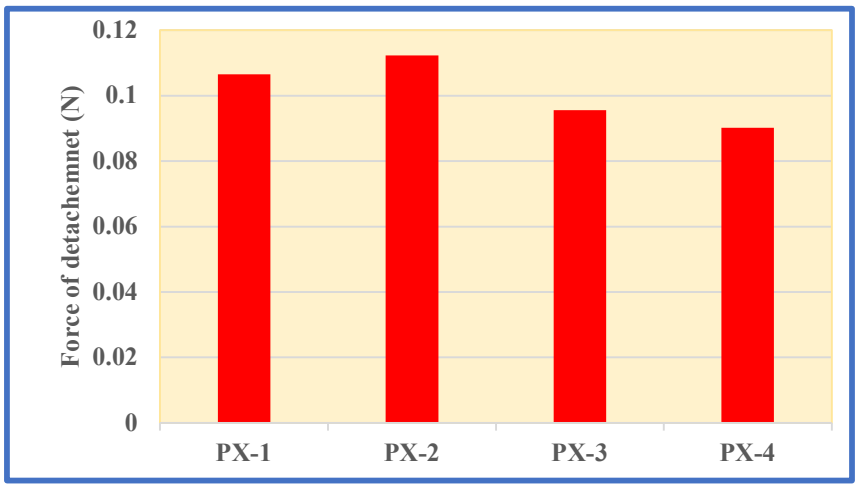


Figure (8). Mucoadhesion force of poloxamer-chitosan in situ gel formulas.

Animal studies and in vivo bioimaging

6-C-CS-LPHNs uptake and retention by bladders wall

We tried to develop a nanoparticle system which have the ability to penetrate the bladder urothelium and pass to underlying tissue in order to targeted the drug to the site of action.

In this experiment, we treated the mouse with 6-C-CS-LPHNs in situ gel, a fluorescence material that after taken up by the bladder wall it may be visualized using confocal laser scanning. Images from CLSM are seen in figure (5). Pictures reveals penetration of nanoparticles of all bladder tissues starting with mucosa passing through lamina propria and detrusor muscle and even Adventitia penetrated by 6-C nanoparticles. We may suggest that CS-LPHNs can be used to deliver the drug for bladder layers.

Also, images reveal distribution of nanoparticles inside the cytoplasm, nucleus and intracellular junction.

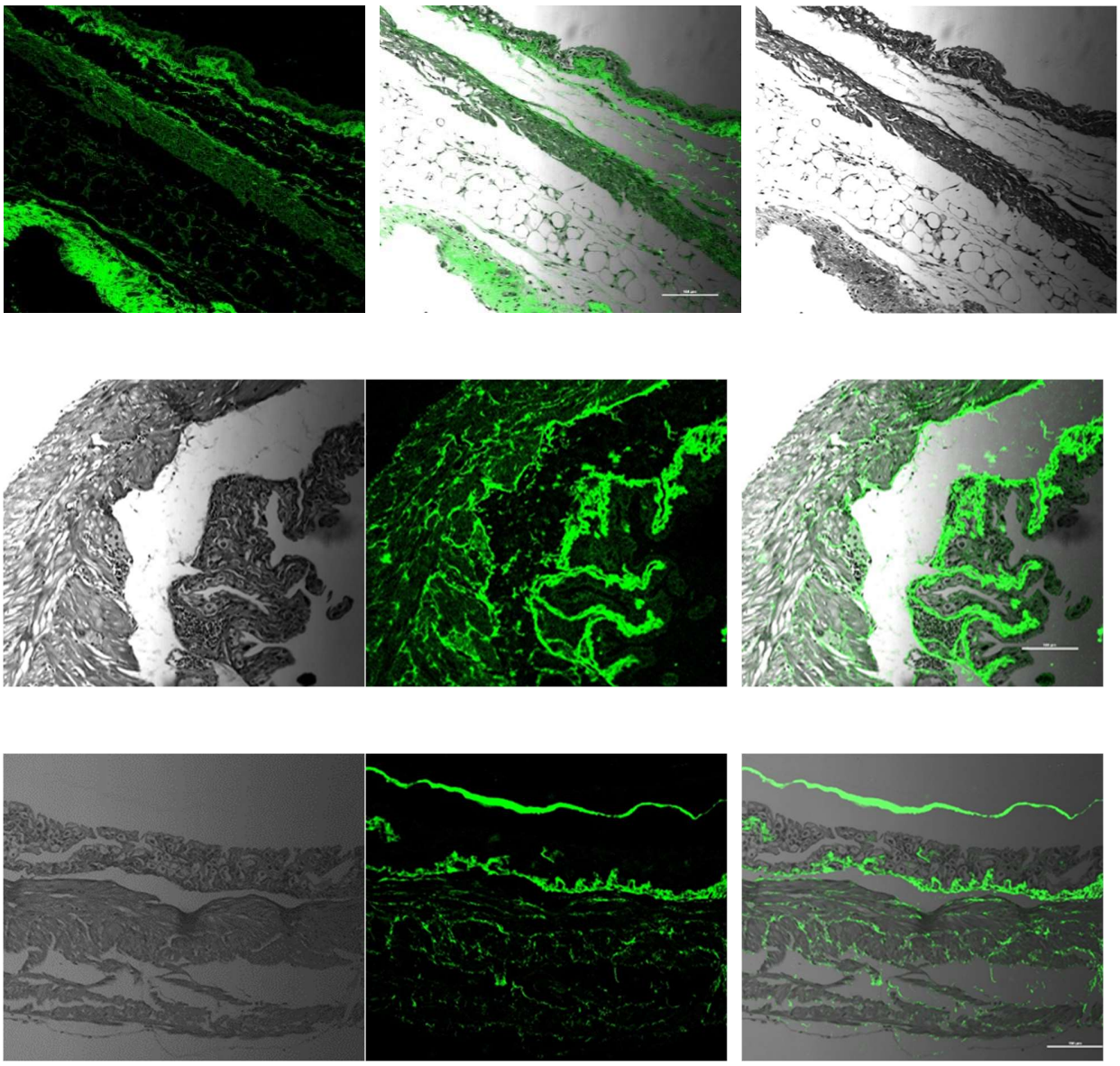


Figure (9). Confocal laser scanning microscope images for cross-section of bladder tissues after treated with 6-C-CS-LPHNs in situ gel for 2hrs.

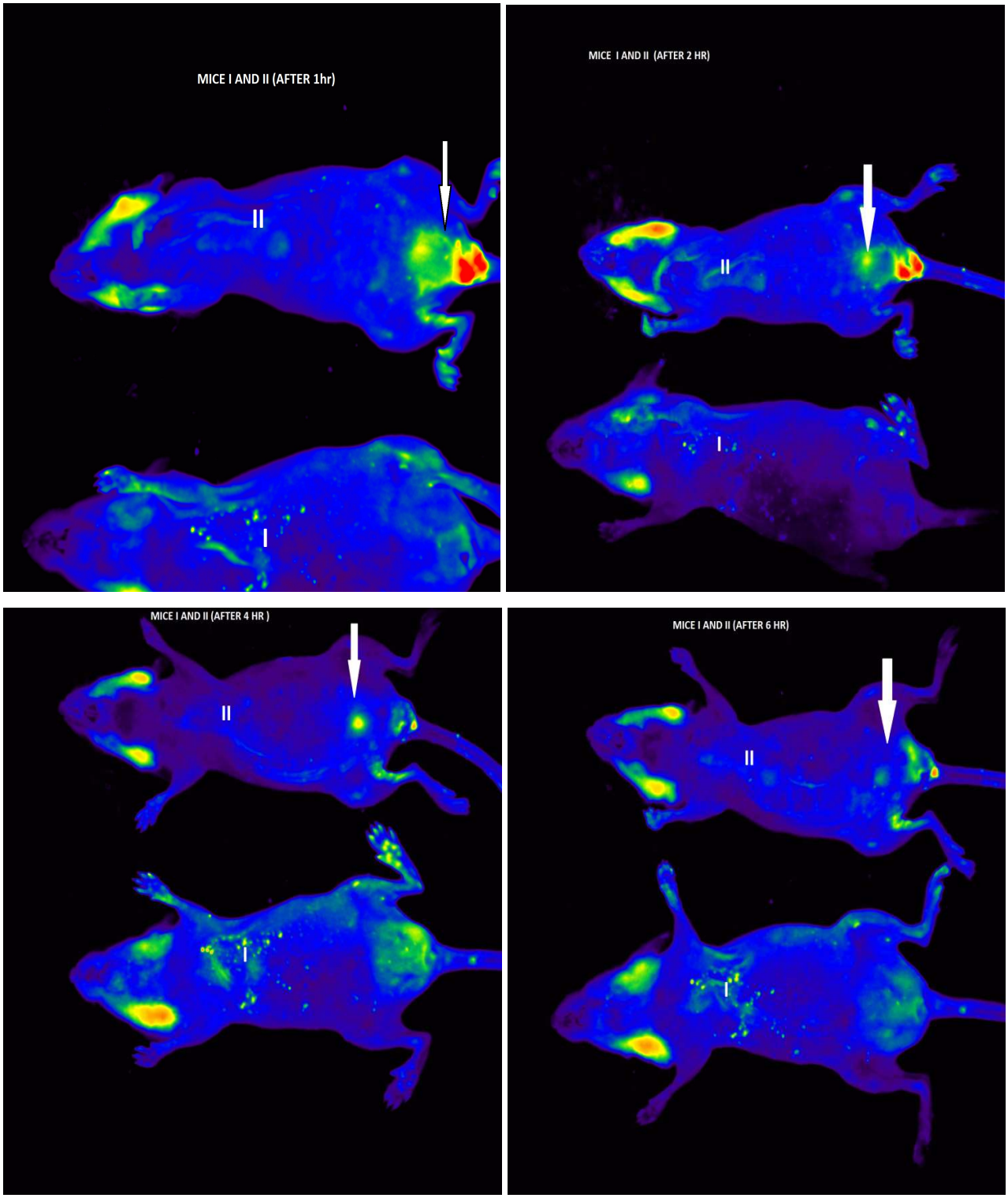
In vivo fluorescence imaging

In order to investigate the potential of such hybrid system as the intravesical drug delivery system for in vivo drug delivery. FITC was used as fluorescence probe for detection of hybrid nanoparticles inside the body. Twelve mice taken these hybrid nanoparticles (FITC-TAC-CS-LPHNs) intravesically, and the fluorescence were measured at a different time interval. The mouse that have (code I) given PBS and consider as control groups.

Six mice were given with FITC-TAC-CS-LPHNs solution (having the code II and III). Pictures shows that solution stay for at least 2hrs in the bladder and successfully urine out of the bladder, and still little fluorescence intensity in the bladder area up to 4hrs which may be attributed to the bladder tissue uptake of FTIC-CS-LPHNs, also, it doesn't show any body distributions to other organs.

Three mice were given the FITC-TAC-CS-LPHNs in situ gel intravesically. Few minutes after injection, the gel shows a spherical shape red dot inside the bladder which indicated the gel formation which start dissolution and pushed out of the bladder gradually up to 3hrs. Thus we can conclude that the residence time of the gel in the bladder was between 2-3hrs and doesn't interfere with the urination process.

The fluorescence activity that seen in the head of the mice due to the hair that doesn't shaved as the whole body.


MICE I AND II (AFTER 1hr)
II
I
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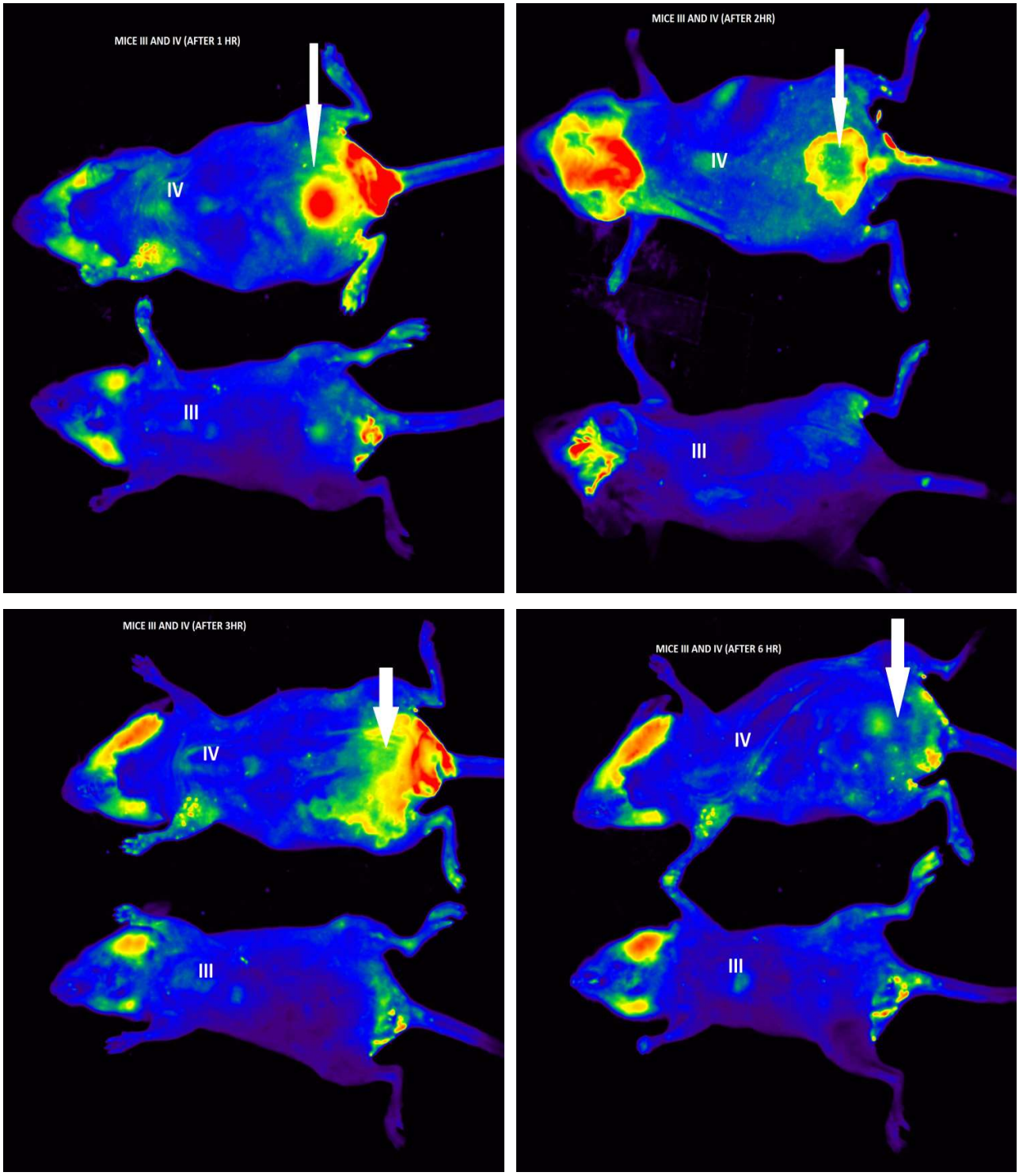

Figure (10). In vivo fluorescence imaging of FITC-CS-LPHNs treated mice. (Mice 1 treated with PB, mice 2 and 3 treated with FITC-CS-LPHNs and mouse 4 treated with FITC-CS-LPHNs in situ gel.)

Conclusion

Bladder pain syndrome remains challenging for treatment due to barriers and limitations of urinary bladder like frequent urination and low penetration capability. Tacrolimus loaded CS-LPHNs may enhance the uptake of Tacrolimus by bladder tissue due to the cell membrane like property. The incorporation of TAC-LPHNs inside Poloxamer\Chitosan thermosensitive in situ gel increased the time of occupancy in the bladder. CLSM images show the retention of dye (coumarin 6) inside the bladder tissue after 2 hrs of installation. The gel shows a spherical shape red dot inside the bladder which indicated the gel formation and start to dissolute and pushed out of the bladder gradually up to 3hrs. thus we can conclude that the residence time of the gel in the bladder is between 2-3hrs. and doesn't interfere with the urination process.

Data show that PX-4 have the least viscosity and work of compression required for injecting the formula inside the bladder and thus may be consider as a selective formula.

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Conflict of interest

None declared.

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