SHORT COMMUNICATION

The effects of topically applied polyNIPAM-based nanogels and their monomers on skin cyclooxygenase expression, *ex vivo*.

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Keywords: nanogel, NIPAM, acrylic acid, stimulus-responsive, pH, temperature, inflammation, COX-2, skin

Abstract

Stimulus-responsive nanogels have potential as carriers for drugs targeting the skin. It is important to estimate the biocompatibility of such materials with the skin since they are directly in contact upon application and may induce irritation or inflammation. In the current work, blank (drug-free) poly*N*-isopropylacrylamide (polyNIPAM), poly(NIPAM copolymerized butyl acrylate) [poly(NIPAM-*co*-BA)], and poly(NIPAM copolymerized with 5% w/v acrylic acid) [poly(NIPAM-*co*-AAc)(5%)] nanogels were dosed onto freshly excised full-thickness porcine ear skin and the effects on the expression of cyclooxygenase-2 (COX-2) determined *ex vivo* by Western blotting. Modulated COX-2 expression was indicative that the material had penetrated the skin and keratinocytes of the viable epidermis. The poly(NIPAM-*co*-BA) nanogel was found to exert a proinflammatory response when applied topically, as reflected by 67% higher COX-2 expression relative to the control treatment (p = 0.0035). The data obtained for the poly(NIPAM-*co*-AAc)(5%) nanogel on the other hand, indicated no significant modulation in the expression of COX-2 (p = 0.1578), suggest the particles are compatible with skin. This was even the case in the presence of co-administered aqueous citric acid solution. Overall the data support the use of the multi-responsive poly(NIPAM-*co*-AAc)(5%) nanogel for triggered or controlled topical drug delivery applications.

1 Introduction

Definitions of nanomaterials have been proposed by various organizations and typically range from 1 - 100 nm dimension (Scientific Committee on Emerging and Newly Identified Health Risks, 2010, US Food and Drug Administration (FDA) Center for Drug Evaluation and Research, 2012). Even though this scale is generally accepted, it is very difficult to clearly define the top end of the range, as there is no scientific evidence to qualify the appropriateness of the 100 nm limit, i.e. no data are available to indicate that a specific size associated with special properties due to the nanoscale. Additionally, FDA has suggested that nanomaterial can also be defined as an engineered material or end product that exhibits properties or phenomena, including physical or chemical properties or biological effects that are attributable to its dimension(s), even if these dimensions fall outside of the nanoscale range, up to one micrometer (US Food and Drug Administration (FDA) Center for Drug Evaluation and Research, 2012, US Food and Drug Administration, 2010).

Drug carriers of nanoscale dimensions were first reported in the early 70s and since then, they have rapidly evolved in the pharmaceutical field (Otto and M. de Villiers, 2009). Nanogels are nanometric scale networks of chemically or physically cross-linked polymer particles (Guterres et al., 2007, Oh et al., 2009). Depending on the chemical nature of the monomers used, nanogels undergo conformational transition in the form of volume collapse in response to external stimuli, such as temperature (Hoare and Pelton, 2004), ionic strength (Neyret and Vincent, 1997) and solvent type (Kaneda and Vincent, 2004). This stimuli-induced behaviour, of absorbing solvated materials into the particles such as drugs under a set of conditions, then releasing them when environmental conditions change, can be beneficial in drug delivery (Lopez et al., 2004, Zhang et al., 2006). Due to their submicron size and multiple-stimuli sensitivity, they have potential to improve methods of drug delivery over conventional formulations such as creams or ointments. To date, limited work has been published on the use of nanogel particles as drug carriers for topical administration, although enhanced penetration of a lipophilic sunscreen (Alvarez-Román et al., 2004), methotrexate (MTX) (Singka et al., 2010) and caffeine (Abu Samah, 2011) have been reported.

In the development of any new drug delivery system, especially involving those at the nanoscale, it is necessary to characterize potential for adverse events. Recent studies have suggested that poly*N*-isopropylacrylamide (polyNIPAM)-based polymers are biocompatible even with exposure for longer durations (up to 96 h) (Naha et al., 2010a, Wadajkar et al., 2009). The cellular internalization of nanoparticles has also been reported (Naha et al., 2010a), a phenomenon supported by Singka who found reduced levels of prostaglandin E_2 (PGE_2) in skin dosed with MTX-loaded nanogel (Singka et al., 2010). Additionally, it was reported that some unreacted (residual) monomers could possibly remain even after being purified, typically in the range of 10 - 1000 ppm for polymers used in cosmetic products (Zondlo, 2002). However, after assessment, the Cosmetic Ingredient Review (CIR) Expert Panel considered the reported levels as safe to be used in cosmetic formulations.

The skin plays a critical role as a part of the human body defense mechanism system. Beneath the cornified stratum corneum (SC) is the viable epidermis which is composed of living cells (keratinocytes) that can exhibit rapid characteristic tissue response to

damage and irritation via inflammation, as well as protective and repair processes (e.g. wound healing) when the irritants get through. The keratinocytes respond to skin irritation and injury by cytokine release and a rapid but transient activation of arachidonic acid metabolism along both the cyclooxygenase (COX) and lipoxygenase pathways (Marks et al., 1998). COX is the key enzyme that is responsible for the conversion of arachidonic acid to prostaglandins. Two main COX isoforms have been identified and are recognized as cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). COX-1 and COX-2 are involved in the biosynthesis of PGE_2, a major prostaglandin involved in epidermal homeostasis and repair. COX-1 is thought to be involved in normal skin homeostasis, whereas COX-2 is important in various responses involving insults to the skin either mechanical or chemical. COX-2 has a short half-life and so the relative levels of its expression can be directly related to the level of pro- or anti- inflammatory stimulus at a particular time point, given that the cells or skin tissues remain viable. This approach has been used in probing the UV-induced inflammation in human keratinocyte (HaCaT) cells (Shibata et al., 2010) and by to determine the anti-inflammatory properties of fish oil in *ex vivo* skin (Thomas et al., 2007).

Exogenous compounds and materials applied to the skin have the potential to be toxic or irritant. In this study, we wanted to examine the propensity of blank (drug-free) nanogels based on *N*-isopropylacrylamide (NIPAM) to induce skin inflammation. One of the early presenting features in such events is the induction of inflammatory processes. In the skin, this involves up-regulation in the biosynthesis of COX-2, which in turn catalyses production of the inflammation mediators, prostaglandins. Monitoring the modulation of short-lived, inducible COX-2 *ex vivo*, can provide a predictive model of *in vivo* inflammation (Zulfakar et al., 2010). To the best of our knowledge, there are no published reports on the propensity of nanogels to induce inflammatory processes in skin. Clearly, if such materials caused adverse skin reactions, it would limit their applicability in a clinical setting. The present study was therefore undertaken to investigate potential inflammatory side-effects following the topical application of the polyNIPAM-based nanogels, and their monomers (Table 1) at the tissue level.

In the current work, Western blot analysis was carried out to quantify the expression of COX-2 in protein extracts from full-thickness *ex vivo* porcine skin following exposure to the nanogels, their corresponding monomers (in view of the potential for residual monomer content of the nanogels) and a pH modulator (aqueous citric acid, CA). Two nanogels of interest were investigated - poly(NIPAM copolymerized butyl acrylate) [poly(NIPAM-*co*-BA)] and poly(NIPAM copolymerized with 5% w/v acrylic acid) [poly(NIPAM-*co*-AAc)(5%)], as both nanogels were shown to enhance the delivery of the loaded permeants across the SC (Singka et al., 2010).

2 Materials and methods

2.1 Materials

AAc (99%), CA anhydrous (99.5%), *N,N′*-methylenebis-acrylamide (MBA), sodium hydroxide (NaOH), Whatman® qualitative filter paper Grade 4, acrylamide/bis-acrylamide (30% solution v/v), ammonium persulfate (APS, ≥98%), aprotinin (≥98%), dithiothreitol (DTT, 1 M in water), ethylene diamine tetraacetic acid (EDTA, 98%),

Hanks' balanced salt buffer (HBSB), leupeptin hydrochloride (≥70%), Monoclonal Anti-β-Actin antibody produced in mouse (clone AC-74, ascites fluid, A 5316), [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] (HEPES, ≥99.5%), phenylmethylsulphonyl fluoride (PMSF, ≥99%), phosphate buffer solution (PBS, pH 7.4), polyoxyethylene-sorbitan monolaurate (Tween® 20), ponceau S and Radio-Immunoprecipitation Assay (RIPA) Buffer were purchased from Sigma-Aldrich (Poole, UK). Cyclooxygenase-2 antibody (COX-2, #4842), anti-rabbit immunoglobulins (IgG) horseradish-peroxidase (HRP)-linked antibodies and positive controls for COX-2 (RAW 264.7 cells lysate, untreated or LPS treated) by Cell Signaling Technology were purchased from New England BioLabs Ltd. (Hitchin, UK). Full range Rainbow® recombinant protein molecular weight marker (12 - 225 kDa) was purchased from GE Healthcare Life Sciences (Little Chalfont, UK) and Bio-Rad protein assay reagent from Bio-Rad Laboratories GmbH (Munich, Germany). MXB autoradiography film (blue sensitive: 18 × 24 cm²) was obtained from Genetic Research Instrumentation Ltd. (Braintree, UK). Marvel original dried skimmed milk was purchased from Chivers Ireland Ltd. (Dublin, Ireland). NIPAM (99%), BA (99%), potassium persulfate (KPS, 99.9%), glass wool, bovine serum albumen (BSA), bromophenol blue (99%, UV-VIS), dimethyl sulfoxide (DMSO, 99%), gentamycin sulfate, glycerol (99%), glycine (99%), *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED, 99%), positive control lysate for COX-2 (Human cells-13 lysate, 250 µg in 0.1 mL), sodium bicarbonate ($NaHCO_3$, 99%), sodium chloride (NaCl, 99.9%), sodium dodecyl sulphate (SDS), tris (hydroxymethyl)methylamine (Tris base, 99.8%), Thermo Scientific SuperSignal® West Dura Extended Duration Substrate, filter paper QL100 (equivalent to Whatman Grade 1), nitrocellulose transfer membrane (Whatman Protran® BA85 with pore size of 0.45 µm) and all other solvents were of analytical grade or equivalent were obtained from Fisher Scientific (Loughborough, UK). Freshly excised porcine ears were obtained from a local abattoir and immersed in iced HEPES-buffered Hanks' balanced salt (HBHBS) solution upon excision, and used within 3 h of slaughtering.

2.2 Nanogel synthesis

PolyNIPAM nanogel synthesis was carried out by a single-step surfactant-free emulsion polymerization reaction. NIPAM, MBA, BA or AAc and de-ionized water (150 mL) were added together in a beaker as outlined in Table 2, and stirred for ~15 min. The mixture was continuously stirred at 300 rpm and immersed in a water bath heated to the polymerization temperature of ~70°C. The flask was continuously purged with nitrogen gas to maintain anoxic conditions, as oxygen could act as a free-radical scavenger that might interfere with the polymerization process. After stabilizing for 30 min at ~70°C, polymerization was initiated by the addition of a hot pre-dissolved persulfate initiator (made-up beforehand in 100 mL of de-ionized water). The color of the solution turned from colorless to sky blue until it reached an ultimate ivory white hue, indicating successful polymerization (Lin et al., 2006, Pelton and Chibante, 1986). The reaction was allowed to proceed for a further 6 h. Once completed, the crude nanogel suspensions were cold-filtered through glass wool and filter paper then subjected to repeated steps of centrifugation [50,000 x g, 20°C for 1 h in a temperature-controlled Beckman Coulter Avanti® J-25 centrifuge (Beckman Coulter Inc., Fullerton, CA)], decantation and re-

dispersion in de-ionized water (5 times). After the final centrifugation/decantation step, the nanogel pellets were stored in a freezer of -20°C for 1 h, then placed into a MicroModulyo 230 freeze-dryer (Thermo Scientific, NY) until completely dry (~48 h). The resulting product was then stored at 2 – 4°C until use. The characterizations of the product nanogels are described elsewhere (Samah et al., 2010).

2.3 Topical application and preparation of skin lysates

The freshly excised full-thickness porcine skin sections were mounted in glass Franz-type cells. The receptor compartment filled with temperature-equilibrated (~32°C), degassed HBHBS-gentamycin (receptor phase). The receptor phase was made up of HBSS (9.7 g), HEPES (6 mg), sodium bicarbonate (0.35 g), and gentamycin sulfate (50 mg) in 1 L de-ionized water. The HBHBS was used as it was proven able to maintain skin viability for 24 h (Bronaugh, 2007), while gentamycin served a role to inhibit or minimize bacterial growth in the receptor phase. A micro magnetic stirrer bar was added into each Franz cell and the complete assembly was placed on a submersible magnetic stirring plate (Variomag, Daytona Beach, FL) set up in a thermostatically controlled water bath (Fisher Scientific, Loughborough, UK), maintained at ~37°C (the core temperature in vivo), providing a skin surface temperature of ~32°C. Skin samples were recovered from the diffusion cells 9 h post-application of the treatments (30 mg, gently massaged) and the areas to which the treatments were applied were excised. They were then cut into small pieces and homogenized using a Silverson® homogenizer (Silverson Machines Ltd., Chesham, UK) in a lysis buffer (RIPA buffer with additional fresh protease inhibitors: PMSF, EDTA, aprotinin, and leupeptin) at 2°C. After 15 min incubation on ice, samples were centrifuged in the Heraeus Multifuge 3 S-R (33,000 x g, 4°C for 15 min), twice in Eppendorf® tubes. The supernatants were stored at -20°C.

2.4 Protein estimation

Total protein concentration in the skin lysates was determined using the Bio-Rad protein assay kit (modified Lowry method) and a standard curve produced using dilutions with a range of 0 - 25 μg mL^{-1} BSA in de-ionized water. The absorbance was determined at 595 nm using a UV spectrophotometer (CECIL Instruments CE2041 series 2000, Cecil Instruments, Cambridge, UK). The protein concentration in each diluted sample was obtained through extrapolation of the standard BSA curve. All samples and standards were prepared in replicates of three (n=3).

2.5 COX-2 denaturation

Skin lysates containing 25 μg of soluble proteins were aliquoted to Eppendorf® tubes and diluted in a 1:1 ratio with 2X Laemmli buffer containing 0.1 M DTT. The tubes were placed in the heating block at 100°C for 5 min, and the samples were cooled down for 5 min and mixed gently at room temperature before subjected to centrifugation (Heraeus Multifuge 3 S-R, 33,000 x g at 4°C for 1 min). The denatured samples were then used straight away.

2.6 Western blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a XCell SureLockTM Mini - Cell (Invitrogen Ltd., Paisley, UK) powered by a Powerpac 300 power pack (Bio-Rad Laboratories Ltd., Hemel Hempstead, UK) following the manufacturer's guidelines. Samples were resolved on 10% SDS-PAGE separating gel and 4% SDS-PAGE stacking gel. The samples prepared were loaded alongside 2.5 μL of Full range Rainbow® recombinant protein molecular weight marker (10 - 250 kDa) and a positive control lysate for COX-2. β-actin served as a loading control. Histograms represent the ratio of the protein of interest against β-actin and the control is 100%. The method used was described previously (Ouitas and Heard, 2010).

2.7 Data analysis

The data were analyzed using Excel 2007 (Microsoft Office, Microsoft Corp., Redmond, WA) and expressed as a mean ± SD. Statistical analysis was performed with InStat® for Macintosh, version 3.00 (GraphPad Software Inc., San Diego, CA). Significant differences and comparisons of the means between paired data were made using Student's *t*-test. For multiple comparisons, a one-way analysis of variance (ANOVA) analysis was carried out with Tukey's post-test to identify statistical significances between groups. Confidence interval was 95% where $p<0.05$ was considered to be significant.

3 Results and discussion

In this assay, the samples were defined as pro-inflammatory if the level of COX-2 expression was significantly greater than the vehicle-treated control, i.e. de-ionized water, and anti-inflammatory if lower than the control. COX-2 expression is associated with inflammatory cascade, thus, any treatments that increased the COX-2 level would be indicative of inflammation in the skin tissue. It would also provide confirmatory evidence that the material had penetrated the skin and keratinocytes.

3.1 Poly(NIPAM-*co*-AAc)(5%) nanogel

The blots obtained for the poly(NIPAM-*co*-AAc)(5%) nanogel, its monomers (NIPAM and AAc), pH modulator (aqueous solution of CA) and control nanogel (polyNIPAM) are shown in Figure 1. There was no significant difference between treatments in comparison to the skin treated with only vehicle (control) ($p = 0.5123$). The poly(NIPAM-*co*-AAc)(5%) nanogel caused a slightly higher COX-2 expression by only 17%, and is considered insignificant ($p = 0.1578$). This promising result suggests that the poly(NIPAM-*co*-AAc)(5%) nanogel is not pro-inflammatory even in the presence of aqueous CA solution (pH modulator). A similar result was exhibited by the polyNIPAM ($p = 0.1458$). The non-inflammatory effect exhibited by the poly(NIPAM-*co*-AAc)(5%) and polyNIPAM nanogels could be possibly due to the absence of any reactive constituents which might induce any interactions with skin components. The results

obtained can be further supported by one of the recent publications, where polyNIPAM nanoparticles were assessed for cytotoxicity effect in a HaCaT cell line which served as a dermal model (Naha et al., 2010a). The authors discovered that the particles were internalized in the cells and predominantly localized in the lysosomes. However, they did not significantly induce cytotoxicity even over a broad concentration range (25 - 1000 μg mL^{-1}). Based on the data in this study, it can be inferred that the poly(NIPAM-*co*-AAc)(5%) nanogel was found not to be pro-inflammatory to the skin.

3.2 Poly(NIPAM-*co*-BA) nanogel

The densitometric analysis of the resulting bands for COX-2 (Figure 2) demonstrated that the poly(NIPAM-*co*-BA) nanogel is pro-inflammatory. The analysis exhibited a marked induction of COX-2 by the nanogel, with COX-2 expression of 67% higher than the control, i.e. skin treated with the vehicle alone ($p = 0.0035$). This was followed by the polyNIPAM with COX-2 level of 18% higher than the control ($p = 0.1458$). Surprisingly, the aqueous solution of NIPAM monomer (1%) was found not to be pro-inflammatory, as its COX-2 expression level was about the same as the control ($p = 0.8905$). Much less intense expression was observed in the skin treated with the saturated aqueous solution of MTX than the skin dosed with other treatment groups. It was confirmed that the COX-2 level was significantly reduced in the skin treated with MTX by about 63% in comparison to the control ($p = 0.0060$).

The data therefore indicate that the poly(NIPAM-*co*-BA) nanogel is pro-inflammatory. The inflammatory effect demonstrated by the nanogel on the skin tissue might be triggered in a similar mechanism as the tissue responding after exposure to an injurious agent (Ghanayem et al., 1985). In addition to that, oxidative stress pathway might also be responsible for the inflammatory reaction. Several nanomaterials had exhibited toxicity both *in vitro* and *in vivo* via induction of oxidative stress by free radical formation at the particle surface (Naha et al., 2010a). Excessive level of free radicals may cause impairment to biological components due to oxidation of lipids, proteins and deoxyribonucleic acid (DNA). It was reported that, nanoparticles of poly(amido amine) (PAMAM) dendrimers had exhibited toxic and inflammatory-like reactions in cells via this pathway (Naha et al., 2010b).

The observed pro-inflammatory response was unexpected but may be explained by several factors, including a hydrophobicity factor. The poly(NIPAM-*co*-BA) was prepared by copolymerizing the NIPAM with the BA co-monomer, which aimed to reduce the lower critical solution temperature (LCST) of the resultant copolymer compared to the polyNIPAM, by increasing its overall hydrophobicity. Thus, the nanogel was more hydrophobic in comparison to the poly(NIPAM-*co*-AAc)(5%), owing to its extra hydrocarbon chain originated from the BA co-monomer (Table 1). In a study conducted aimed to investigate cell attachment and detachment control with temperature-induced alteration of surface properties with grafted polyNIPAM copolymers, it was reported that the polymers caused deterioration of cellular metabolic functions at lower temperatures, when they were in a hydrophilic state (Tsuda et al., 2004). This condition is suggested due to cell- and protein- adhesion behavior of the polymers. Individual functional groups have been demonstrated to affect protein adsorption, cellular response and cell-biomaterial interactions (Lynch et al., 2005, Lindman et al., 2007). Surfaces

displaying methyl groups (-CH_3) bound to proteins more firmly in comparison to surfaces displaying hydroxyl groups (-OH) (Tengvall et al., 1998). This interaction may trigger immune response, as shown in another independent study, where the methyl groups were found to trigger immune response by increasing the adhesion of inflammatory cells (Lindblad et al., 1997). In the current study, the BA region of the poly(NIPAM-*co*-BA) possessed methyl groups originating from the butyl hydrocarbon, whereas the poly(NIPAM-*co*-AAc)(5%) possessed carboxyl groups originating from the AAc residues.

Apart from the above factors, the samples obtained for this study were obtained from the *in vitro* experiment with static diffusion cells. A potential disadvantage of the static diffusion cells is that accumulation of penetrants may occur both in the skin and receptor chambers in comparison to *in vivo* models with more complex systems (e.g. blood clearance and presence of enzyme activities) (Chilcott et al., 2001). Thus, the likelihood to produce the observed effect is higher in the *in vitro* model compared to the *in vivo* model. In that context, the data from our *ex vivo* set-up may be viewed as a worst case scenario.

Referring to the *ex vivo* study of the MTX-loaded poly(NIPAM-*co*-BA) nanogel, it was demonstrated that the nanogel was capable of delivering MTX across the epidermis in levels that significantly reduced the biosynthesis of PGE_2, a key inflammation mediator and product of COX-2 enzymes (Singka et al., 2010). Thus the observed reduced level of PGE_2 was due to the enhanced delivery of MTX, which appeared to overwhelm the pro-inflammatory effect produced by the nanogel.

4 Conclusion

Poly(NIPAM-*co*-AAc)(5%) nanogel provided no modulation in COX-2 expression, suggesting it is skin-compatible, hence suitable for multi-responsive poly(NIPAM-*co*-AAc)(5%) triggered drug delivery applications. On the other hand, poly(NIPAM-*co*-BA) nanogel was found to significantly induce COX-2 reaction.

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Legends to Figures

Figure 1. Western blotting and densitometric analysis of COX-2 protein expression for poly(NIPAM-*co*-AAc)(5%). Porcine full-thickness skin was treated for 9 h with the following - control (de-ionized water); NIPAM monomer (1% w/v); AAc monomer (0.05% v/v); aqueous solution of CA (5% w/v); polyNIPAM; poly(NIPAM-*co*-AAc)(5%); poly(NIPAM-*co*-AAc)(5%) followed by CA solution; and MTX – negative control. Results were normalized using β-actin and level in control was arbitrarily assigned a value of 100%, (n=3, ± SD). $p<0.5123$ between treatment groups.

Figure 2. Western blotting and densitometric analysis of COX-2 protein expression for poly(NIPAM-*co*-BA). Porcine full-thickness skin was treated for 9 h with the following - control (de-ionized water); NIPAM monomer (1% w/v); polyNIPAM; poly(NIPAM-*co*-BA); and MTX (negative control). Results were normalized using β-actin and level in control was arbitrarily assigned a value of 100% (n=3, ± SD). * indicates $p = 0.8905$, *vs.* control; ** indicates $p = 0.1458$, *vs.* control; *** indicates $p = 0.0035$, *vs.* control and **** indicates $p = 0.0060$, vs. control.

Legends to Tables

Table 1. Physicochemical properties of NIPAM, AAc and BA monomers (MW – molecular weight; cLog *P* – partition coefficient for n-octanol/water).

Table 2. Treatments applied for the protein immunoblot study.

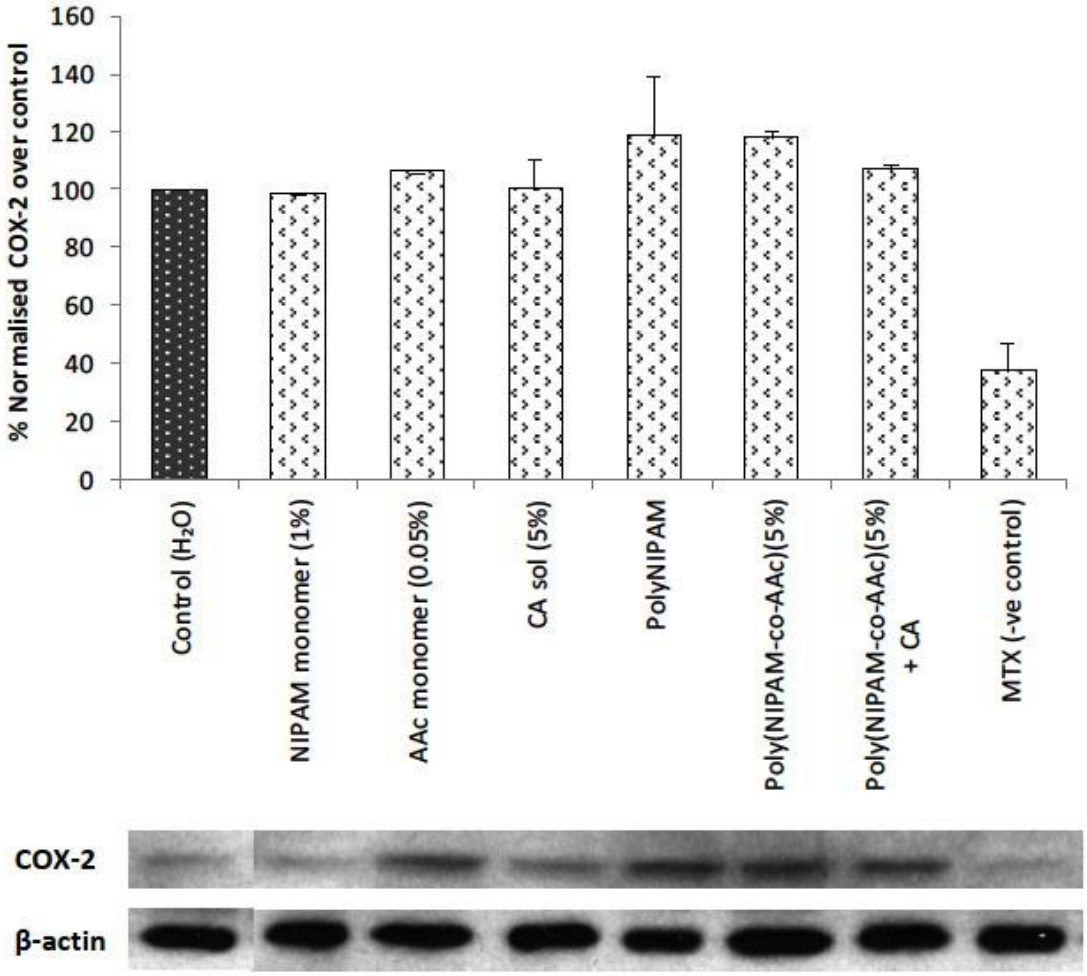

% Normalised COX-2 over control
160
140
120
100
80
60
40
20
0
Control (H2O)
NIPAM monomer (1%)
AAc monomer (0.05%)
CA sol (5%)
PolyNIPAM
Poly(NIPAM-co-AAc)(5%)
Poly(NIPAM-co-AAc)(5%) + CA
MTX (-ve control)
COX-2
β-actin

Fig 1

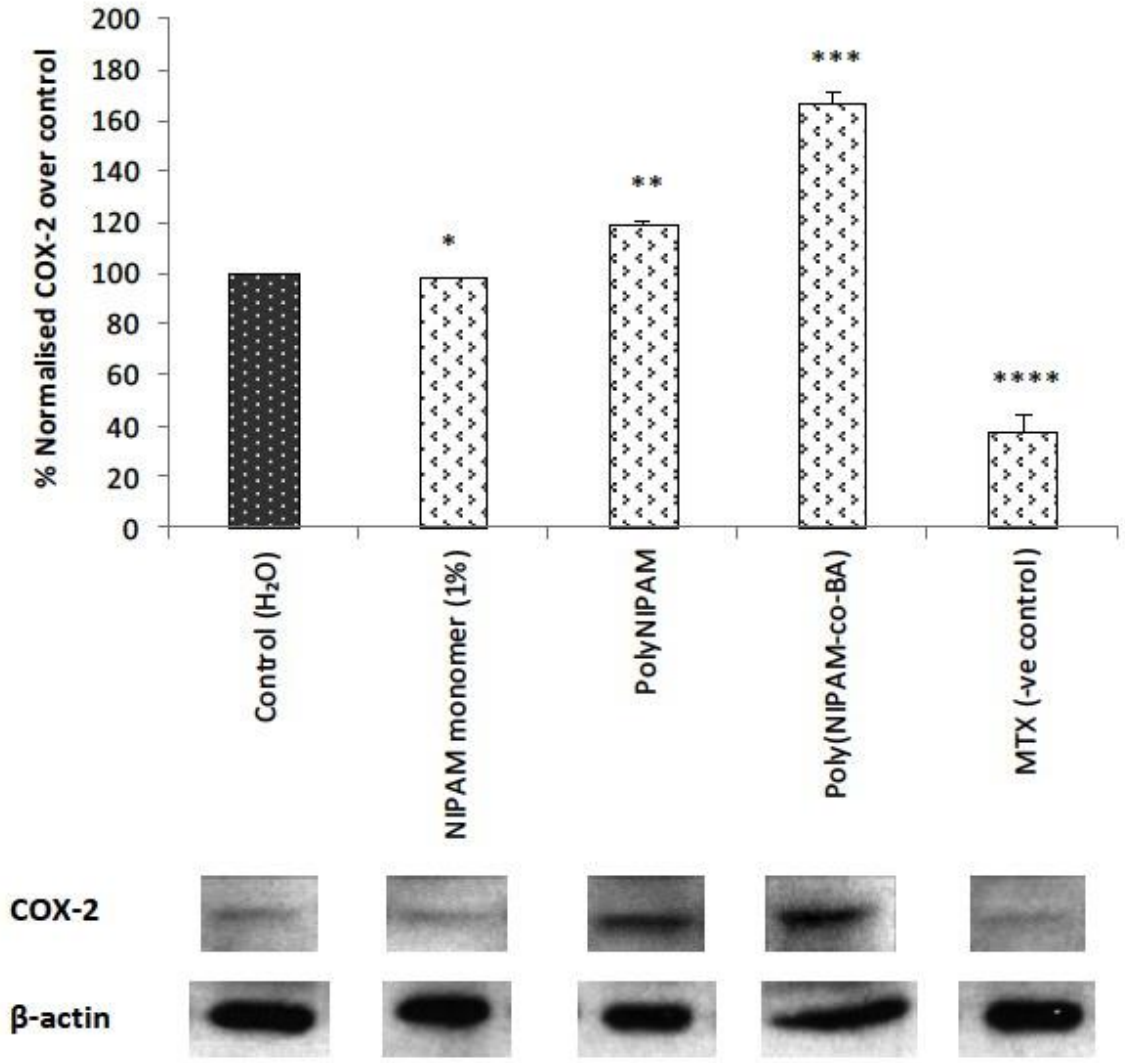

% Normalised COX-2 over control
200
180
160
140
120
100
80
60
40
20
0
*
**
***
****
Control (H2O)
NIPAM monomer (1%)
PolyNIPAM
Poly(NIPAM-co-BA)
MTX (-ve control)
COX-2
β-actin

Fig 2

Table 1

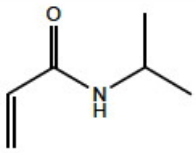
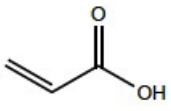
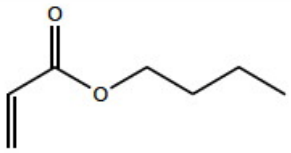
	NIPAM	AAc	BA
Chemical Structure			
Chemical Formula	$C_6H_{11}NO$	$C_3H_4O_2$	$C_7H_{12}O_2$
MW	113.16	72.06	128.17
cLog *P*	0.28	0.31	2.36

Table 2

	Dosing Regimen	Dose (µL)
(i)	Aqueous solution of NIPAM monomer (1% w/v, pH 6.68 ± 0.03) alone	500
(ii)	Aqueous solution of AAc monomer (0.05% v/v, pH 3.10 ± 0.01) alone	500
(iii)	Aqueous solution of CA (5% w/v, pH 1.91 ± 0.01) alone	200
(iv)	Swollen polyNIPAM nanogel alone	500
(v)	Swollen poly(NIPAM-*co*-AAc)(5%) nanogel alone	500
(vi)	Swollen poly(NIPAM-*co*-AAc)(5%), followed by aqueous solution of CA (5% w/v)	500 + 200
(vii)	De-ionised water alone	500
(viii)	Aqueous saturated solution of MTX (pH 4.69 ± 0.10) alone	500
(x)	Swollen poly(NIPAM-*co*-BA) nanogel alone	100