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Novel pH-sensitive interpenetrated network polyspheres of polyacrylamide-*g*-locust bean gum and sodium alginate for intestinal targeting of ketoprofen: *In vitro* and *in vivo* evaluation

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ABSTRACT

In this report, novel pH-sensitive interpenetrated network (IPN) polyspheres were developed utilizing polyacrylamide-g-locust bean gum (PAAm-g-LBG) in combination with sodium alginate (SA) to achieve intestinal targeted delivery of ketoprofen. PAAm-g-LBG was synthesized under microwave irradiation wherein ceric ammonium nitrate was used as reaction initiator and then conversion of PAAm-g-LBG as pH-sensitive copolymer was carried out by alkaline hydrolysis. The PAAm-g-LBG copolymer was characterized through ^{1}H-NMR, FTIR and elemental analysis. The IPN polyspheres exhibited pH-depended swelling or de-swelling with the alteration of surrounding pH. The *in-vitro* release of drug from IPN polyspheres was found to be higher (≈ 90%) in phosphate buffer of pH 7.4 in comparison with that in pH 1.2 buffer (10.6%). The *in-vivo* pharmacokinetic, anti-inflammatory screening and stomach histopathology studies performed on Wistar rats revealed pH sensitivity of IPN polyspheres where ketoprofen was successfully targeted to small intestine resulting in reduced side effects of ketoprofen like ulcer formation, erosion of gastric mucosa and hemorrhages.

1. Introduction

Release controlled multi-unit dosage forms like microspheres, nanoparticles, pellets and microbeads are gaining great importance in comparison with unit dosage forms for oral drug delivery because of various benefits [1,2]. Usage of such dosage forms can be more extended when these systems can respond to environment around. pH-sensitive dosage forms are interesting alternatives for intestinal targeting of drugs [3] and polypeptides through oral route [4,5]. The pH-sensitivity can be attributed to any polymer by the existence of weak acidic/ basic groups; swelling characteristics of such polymers is imparted by functional group's ionization, which is dependent on external medium pH in which polymer is exposed. Various reports are available in the literature on development of pH-sensitive drug delivery systems for oral administration [6].

Grafting is a prominent means of altering biopolymer structure and properties, which improve the stability, heat resistance and metal ion bonding capability; hence grafted polysaccharides are extensively useful for industrial applications [7–10]. So as to overcome reduced biological activity and enhance mechanical strength, synthesis of new materials called interpenetrated networks (IPNs) was carried out. IPN is defined to be combination of two or many polymers existing as a network, which are synthesized and/or cross-linked when both the polymers are present at a time resulting in enhanced mechanical firmness and biological activity. These IPNs have recently gained prominence in controlled and targeted drug delivery field [11].

Locust bean gum (LBG) contains β-(1,4)-D-mannose units where substitution of each of fourth mannose units with a small side chain constituting 1,6 linked α-galactose sugar. It is immensely utilized in food industry as stabilizing agent for modifying viscosity, also in textile industry due to film-forming capability. It is having applications in delivery of drugs and tissue engineering [12,13]. Sodium alginate (SA)

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is composed of 1,4–linked β-D-mannuronic acid and α-L-guluronic acid residues, which can be extracted from brown seaweed and marine source algae [14]. SA produces distinct gels possessing divalent or trivalent cations. Formation of stable network can be achieved with combination of SA and glutaraldehyde (GA); hence, the combination has been found useful in controlled drug delivery [15,16].

However, we did a decent review and understood that, there exists no work on pH-sensitive IPNs of PAAm-g-LBG and SA for application in small intestinal targeted drug release. Subsequent to our earlier reports generated on graft copolymers and their IPNs application in drug delivery [17–19], we now herein summarize, synthesize, develop and evaluate the novel IPN polyspheres of pH-sensitive PAAm-*g*-LBG and SA to target ketoprofen to the small intestine.

Ketoprofen is a non-steroidal anti-inflammatory drug used to treat musculo-skeletal and joint disorders. Half-life of ketoprofen is only 2 h. After administering orally, there occur adverse events like ulceration, hemorrhage, *etc* in stomach. Hence, controlling ketoprofen release without its release in stomach is required for effective treatment and patient compliance [20].

The objective of current research was synthesis of pH-sensitive IPN polyspheres utilizing PAAm-*g*-LBG and SA to target ketoprofen to the small intestine. These IPN polyspheres possess $-COO^-$ functional groups, hence, unionization occurs at stomach pH resulting in lower drug release; in other way, ionization of polyspheres occurs in small intestinal pH and as a result of this, drug release is increased in intestine, which in reduces gastric side effects of ketoprofen.

2. Experimental

2.1. Materials

Ketoprofen was obtained as gift sample from Rhone-Poulenc (Mumbai, India). Sodium alginate, locust bean gum, ceric ammonium nitrate, acrylamide (AAm), potassium dihydrogen phosphate, acetonitrile (HPLC) and methanol (HPLC) were purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Sodium hydroxide, calcium chloride dihydrate and Glutaraldehyde (25% w/v) were procured from SD fine Chemicals (Mumbai, India). Throughout the study, distilled water and analytical grade chemicals were used. As per CPCSEA guidelines approval for animal study protocols from Institutional Animal Ethics Committee was obtained (Reg. No. 1076/C/07).

2.2. Synthesis of pH-sensitive PAAm-g-LBG copolymer

Grafting of polyacrylamide on locust bean gum (PAAm-g-LBG) was carried out utilizing microwave assisted irradiation process [21]. In a reaction vessel, two grams of LBG was soaked in 70 ml double distilled water at temperature of 40 °C overnight by stirring continuously at 50 rpm. The required amount of acrylamide monomer (8.53 g) and ceric ammonium nitrate (CAN; 0.5 g) were dissolved in 10 ml distilled water separately and the same were added to LBG solution. Reaction mixture was then irradiated using microwave oven (GMG 17E 07 SLGX, Godrej, Mumbai, India). At various time intervals, the irradiation was interrupted when the reaction mixture started boiling. This irradiation process was followed by cooling till the conversion of polymer solution into gel took place. Further reaction mixture was kept still for a period of 1 h so as to get the reaction completed. Then the mixture was added to methanol and retained in the same for 24 h. Excess methanol as well as distilled water were added to reaction mixture, filtered and subjected for drying at 50 °C for 12 h. The obtained copolymer was then pulverized and stored in a container which is free from moisture.

Further, in order to transform the above graft copolymer into pH-sensitive graft copolymer, addition of precisely weighed quantity (2 g) of PAAm-g-LBG to 100 ml of 0.9 M sodium hydroxide solution was done with continuous stirring at 100 rpm, where the reaction was continued to occur for one hour at 75 °C in a thermostatic water bath. Then the product was brought down to room temperature, mixed with 200 ml of methanol and kept aside for 12 h to remove excess water. Then thorough cleaning of the product was done by using methanol and dehydrated overnight at 50 °C and preserved in a desiccator.

The percentage grafting efficiency of graft copolymer was determined by means of following equation:

$$\%\text{Grafting Efficiency} = \left(\frac{W_1 - W_0}{W_2}\right) \times 100 \quad (1)$$

where, W_0 = weight of native polymer; W_1 = weight of grafted copolymer; W_2 = weight of acrylamide.

2.3. Characterization

2.3.1. Fourier transform infrared (FTIR) spectroscopy

Using FTIR spectrophotometer (Nicolet, Model Magna 550, USA), the native LBG, PAAm-g-LBG and hydrolyzed PAAm-g-LBG were characterized by potassium bromide pellet technique. The sample and potassium bromides were triturated and pressed into a pellet followed by scanning within the range of 400 and 4000 cm^{-1}.

2.3.2. Proton nuclear magnetic resonance (^{1}H-NMR) analysis

The proton NMR studies were carried out on native LBG, PAAm-g-LBG and acrylamide. Internal standard used was tetramethylsilane (TMS). After samples were dissolved in dimethylsulfoxide (10 mg/dm^3) at 60 °C, spectra derivation was carried out utilizing Varian Spectrophotometer (Mercury Plus 300 MHz NMR, WA, USA).

2.3.3. Elemental analysis

Estimation of percentages of carbon, hydrogen and nitrogen helps in the confirmation of grafting reaction. With the help of CHN analyzer (Flash EA 1112, Thermo Finnigan, Italy), the estimation of these elements was carried out for native LBG, PAAm-g-LBG and hydrolyzed PAAm-g-LBG.

2.4. Formulation of IPN polyspheres

The polymeric solutions as summarized in Table 1 were prepared using double distilled water separately. Accurately weighed amount of ketoprofen was added to polymeric solutions and kept for stirring to attain homogenous dispersion. Hypodermic syringe was loaded with 20 ml of dispersion and extruded slowly into calcium chloride solution through #23 needle under continuous mixing condition. Then the separation and continuous washing of resultant ionically cross-linked IPN polyspheres was done in distilled water. The polyspheres were dried in ambient condition for a day and then at 40 °C for 10 h. The polyspheres were then subjected for covalent crosslinking using glutaraldehyde (GA) added with 1 N HCl at 50 °C for half an hour. Finally, dual crosslinked IPN polyspheres were thoroughly washed using distilled

Table 1
Composition of pH-sensitive IPN polyspheres.

Codes	H-PAAm-g-LBG (% w/v)	SA (% w/v)	LBG (% w/v)	Ketoprofen[a] (% w/w)	$CaCl_2$ (% w/v)	GA[a] (% w/w)
L1	–	4	–	20	5.0	–
L2	1	3	–	20	5.0	–
L3	1.5	2.5	–	20	5.0	–
L4	2.0	2.0	–	20	5.0	–
L5	1.5	2.5	–	40	5.0	–
L6	1.5	2.5	–	20	10	–
L7	1.5	2.5	–	20	10	4
L8	1.5	2.5	–	20	10	6
L9	1.5	2.5	–	20	10	8
L10	–	2.5	1.5	20	10	8

[a] % w/w of dry polymer.

water to eliminate unreacted GA. The polyspheres were dried overnight at 40 °C and preserved in sealed container. Detailed composition of IPN polyspheres with variable polymer combinations are depicted in Table 1.

2.5. Evaluation of IPN polyspheres

2.5.1. Scanning electron microscopy (SEM)

Surface characteristics of IPN polyspheres were evaluated by SEM at a voltage of 10–40 kV. The polyspheres were sputter coated with platinum before observing in scanning electron microscope (JEOL, JSM-6360, Kyoto, Japan).

2.5.2. Determination of size

Average size for a minimum of 100 polyspheres from every batch was determined at 0.001 mm accuracy using a digital micrometer (MDC-25S Mitutoyo, Tokyo, Japan).

2.5.3. Drug entrapment efficiency (DEE)

Each set of IPN polyspheres were weighed accurately and imbibed in 50 ml pH 7.4 phosphate buffer at a temperature of 37 °C for a duration of 12 h. Further, soaked polyspheres were triturated and heated gently for 30 min. The dispersion was centrifuged at 2000 rpm for 15 min to extract the drug in supernatant fluid. UV–vis Spectrophotometer (UV-1800, Shimadzu, Japan) was used at a wavelength of 260 nm to measure absorbance of fluid and to calculate DEE. The polyspheres prepared without drug were used as blank.

2.5.4. Thermogravimetric analysis (TGA)

The TGA was conducted on native SA, grafted LBG and L9 IPN polyspheres in dynamic nitrogen atmosphere (50 ml/min flow rate) at 10 °C/min heating rate and 0–600 °C temperature range using a microcalorimeter (DuPont-9900, USA).

2.5.5. Differential scanning calorimetric (DSC) estimation

DSC study was conducted on plain ketoprofen, blank IPN polyspheres (no drug) and optimized IPN polyspheres (with drug). Heating was carried out within the temperature range of 0–300 °C at 10 °C/min heating rate in the presence of nitrogen atmosphere using differential scanning calorimeter (DSC Q20 V24.4 Build116, TA Instruments, USA).

2.5.6. X-ray diffraction (XRD) analysis

The X-ray diffractograms were obtained in the 2θ range 0–90° using X-ray diffractometer (Rigaku Miniflex II, Japan) for plain ketoprofen, blank IPN polyspheres and optimized polyspheres.

2.5.7. Pulsatile swelling study

For confirming the pH-sensitivity of IPN polyspheres, pulsatile swelling investigation was conducted on polyspheres those were formulated using graft copolymer and native polymer. In 25 ml each of 1.2 and 7.4 pH buffer solutions, appropriately weighed polyspheres (20 mg) were placed at 37 °C. As a result, shrinkage and swelling of polyspheres respectively was observed with alteration in the pH of solution from 1.2–7.4. The polyspheres were separated from buffer solutions at specific intervals of time and remaining surface liquid was removed without affecting the polyspheres. Weight variation due to swelling and shrinking of polyspheres was determined using an electronic balance (Model BL-220H, Shimadzu, Japan). Then % swelling (Q) calculation was done using the formula mentioned below:

$$Q = \frac{W_2 - W_1}{w_1} \times 100 \quad (2)$$

where, W_1 is dry polyspheres weight and W_2 is the swollen polyspheres weight.

2.5.8. In vitro drug release investigation

In-vitro drug release study was performed through dissolution testing apparatus (Electrolab TDT-08 P, (USP), Mumbai, India). Buffer solutions (900 ml) of different pH were employed as medium for dissolution at 100 rpm and 37 ± 0.5 °C. At particular time points, 5 ml each of sample was withdrawn and same volume of corresponding buffer solutions were added. The samples were passed through membrane filter (0.45 μm) and drug concentration in the sample was determined using UV–vis Spectrophotometer (UV-1800, Shimadzu, Japan) at 260 nm wave length after required dilutions.

2.5.9. In vivo pharmacokinetic assessment

Based on *in-vitro* performance, L9 IPN polyspheres were selected to carry out pharmacokinetic assessment. Wistar rats were separated into two groups (n = 6) and kept under fasting for 12 h. The rats of first group were orally administered with plain drug (18 mg/kg added to 0.5% w/v sodium carboxymethylcellulose (NaCMC) solution); to other group, drug loaded L9 IPN polyspheres in an amount equal to 18 mg/kg of drug in 0.5% w/v NaCMC solution were administered orally. At respective time slots, blood samples were withdrawn from orbital sinus of the rats and centrifuged at 3000 rpm using centrifuge (C24 Centrifuge, Sigma, Germany) for 15 min to separate plasma. The plasma samples were preserved at −72 °C for drug assessment using HPLC.

Plasma concentration of drug was assessed by means of modified reverse phase (RP) HPLC technique [22] using HPLC system (Shimadzu, Japan) that consists of two pumps, variable-wavelength UV/vis detector, system controller, LC Solution software and an auto-injector. RP C18 column (Genesis, USA; particle size 5 × 250 × 4.6 mm; maintained at 25 °C) was utilized. Acetonitrile and potassium dihydrogen phosphate buffer (pH 3, 10 mM) mixture (50:50) was the mobile phase. The flow rate was 1 ml/min, 50 μl was injection volume and maximum wave-length was 260 nm. To 90 μl of plasma sample, 10 μl of lercanidipine solution (50 μg/ml) as internal standard was added and the mixture was mixed. Again after addition of cold acetonitrile (300 μl), the mixture was stirred for 2 min. It was then centrifuged for 10 min at 10,000 rpm and 4 °C and the resultant buoyant fluid was examined by HPLC. Using the calibration curve, plasma concentration of ketoprofen was determined. Retention time of ketoprofen and internal standard (lercanidipine) was determined as 9.8 and 16.3 min respectively. The pattern of two peaks was proper and was free from plasma matrix intervention at retention time of any of the peaks. The method was validated for precision (RSD: < 15%) and accuracy (< 15%) and recovery was found to be > 82%. The plasma concentration data was represented as mean ± SD and various parameters in pharmacokinetic evaluation like AUC, C_{max}, T_{max}, $t_{1/2}$ and K_e were assessed by PK Solutions 2.0™ software. Pharmacokinetic determination was ascertained by student's *t*-test (Graph Pad Prism Software) and '*p*' value lower than 0.05 was given consideration of being statistically significant.

2.5.10. Anti-inflammatory activity

Segregation of Wistar rats was carried out into three groups where half a dozen of rats in each group were acclimatized to laboratory environment with surplus food and water. Before commencement of experiment, rats were fasted overnight; however water was allowed. One ml (0.5% w/v) of NaCMC solution was given to Group I (control) rats, ketoprofen equal to 18 mg/kg body weight was given to Group II (standard) rats and drug loaded L9 IPN polyspheres equal to drug 18 mg/kg body weight were given to Group III (test) rats by oral means [23]. Carrageenan-induced paw edema technique was adapted to assess anti-inflammatory effect. Inflammation was made by means of 1% w/v carrageenan (0.1 ml) injection in sub-plantar region of rat's left hind paw. Prior to half an hour of carrageenan injection, oral administration of plain drug and L9 IPN polyspheres was performed. Mercury displacement technique in plethysmometer was used to measure rat's paw volume, where percentage decrease in edema was determined [24].

2.5.11. Stomach histopathology

After anti-inflammatory screening, isolated stomach tissues from sacrificed rats were cleaned three times using normal saline and then preserved in 10% formalin till further usage. Stomach tissues were subjected to several changes upon dehydrating by means of ethanol and xylene as clearing solvent. Then specimens were sliced to sections of 5 µm thickness and they were stained using hematoxylin and eosin (H& E) to examine using binocular light microscope (CXRIII, Labomed, Mumbai, India) and pictures were captured [25].

3. Results and discussion

3.1. Synthesis of pH-sensitive PAAm-g-LBG copolymer

PAAm-g-LBG copolymer synthesis was carried out by microwave assisted method. When microwave irradiation was employed along with CAN, production of free-radical sites on LBG backbone occurs. CAN, being electron deficient captures electrons from −OH groups of LBG towards formation of a new Ce-O bond. Ce-O bond formed was polar when compared with O−H bond and can be easily broken when exposed to microwave irradiation to create free radical sites on LBG backbone. Thus formed free radical sites serve as point of growth for graft chains [26]. During alkaline hydrolysis of PAAm-g-LBG, $-CONH_2$ groups of polyacrylamide were transformed into −COONa groups and this imparts pH-sensitive property to PAAm-g-LBG copolymer (Fig. S1, as shown under supplementary information). The synthesized copolymer grafting efficiency was found to be 95.86%.

3.2. Characterization

3.2.1. FTIR analysis

FTIR spectra of LBG, grafted LBG and hydrolyzed PAAm-g-LBG are presented as Fig. S2 under supplementary information. For neat LBG (Fig. S2A), there was observation of peaks at 3415 cm^{-1} and 2924 cm^{-1} because of −OH stretching and asymmetric stretching of $-CH_2$ groups in cyclic aldehydes respectively. It was due to carbonyl group deformation, a peak at 1617 cm-^{1} was obtained, the peak at 1415 cm^{-1} was attributed to −CH and $-CH_2$ in-plane bending. Because of alcoholic groups and C–O stretching of pyranose ring, there were peaks at 1119 cm^{-1} and 618 cm^{-1} respectively. But in case of PAAm-g-LBG copolymer (Fig. S2B), there was observation of peaks at 3414 cm^{-1} and 3245 cm^{-1} because of merger of N−H stretching of amides and O−H stretching of hydroxyl groups respectively. The 2939 cm^{-1} peak was resultant of C–H stretchings present in alkyl chains. Peaks at 1638 cm^{-1}, 1618 cm^{-1} and 1414 cm^{-1} correspond to primary amide groups on PAAm-g-LBG. Thus, the grafting reaction can be endorsed by FTIR analysis. Whereas, in the spectrum of alkaline hydrolyzed PAAm-g-LBG (Fig. S2C), a peak at 3415 cm^{-1} related to stretching vibrations of −OH groups, peak at 2964 cm-^{1} was resultant of aliphatic −CH stretching and peaks at 1618, 1637 and 1416 cm^{-1} were obtained because of COO- groups. Peak at 3245 cm^{-1} which was seen in grafted LBG was not observed in hydrolyzed PAAm-g-LBG and hence hydrolysis of PAAm-g-LBG was confirmed with N−H stretching absence. The similar results are also observed in the published literature [27,28].

3.2.2. Proton NMR spectroscopy

The ^{1}H-NMR data (Fig. S3, as shown under supplementary information) of acrylamide (AAm), LBG and PAAm-g-LBG suggests that the ^{1}H shifts for TMS (internal standard) was at ˜ 0 and for DMSO (solvent) was at ˜ 2.5 ppm. AAm exhibited the ^{1}H shift at ˜3.4 ppm because of CH(NH) groups and ^{1}H shifts at ˜5.6 is due to CH_2 and at ˜6.2 ppm was because of CH group; whereas in AAm, $CONH_2$ protons were resonated at ˜7.2 and ˜7.6 ppm. The LBG exhibited ^{1}H shifts at ˜1.2 and ˜2.4 ppm, attributed to the protons in CH_3C and CH_3CO_2 groups of LBG. A broad resonance recorded in the range of ˜2.8 and ˜3.4 ppm was because of protons present in CH_3O, CH_2, CH and OH groups. In case of PAAm-g-LBG, along with ^{1}H shifts of LBG, there was observation of a new signal at ˜7.5 ppm, which may be due to protons of $CONH_2$ groups of PAAm appeared on LBG after grafting. This substantiates grafting reaction of PAAm on LBG. Such results were also seen for polyacrylamide-g-poly(vinyl alcohol) copolymer [29–31].

3.2.3. Elemental analysis

Elemental analysis results are summarized in Table S1 under supplementary information. The presence of 0.44% nitrogen, 38.55% carbon and 6.72% hydrogen were observed with LBG. From this, it was understood that there were small traces of proteins in LBG. On the contrary, 12.29% of nitrogen, 37.63% carbon and 6.24% hydrogen were observed in PAAm-g-LBG, which might be because of existence of polyacrylamide's $-CONH_2$ groups on LBG backbone after grafting. While in hydrolyzed PAAm-g-LBG copolymer, nitrogen content was reduced to 3.58%, which correlated with $-CONH_2$ group's transformation into −COONa groups at the time of alkaline hydrolysis. This confirms grafting and alkaline hydrolysis process. Similar results are also reported earlier [32].

3.3. Preparation of IPN polyspheres

When polymer solution in which drug was dispersed is extruded into solution of $CaCl_2$ solution (Ca^{2+}), ionic crosslinking occurs between two different SA strands immediately and at the same time replacement of sodium ions of SA at carboxylate group by calcium ions and conjugation of SA chain with calcium ion to form a linkage occurs. Thereby calcium ions were kept together with two SA chains resulting in formation of spherical shaped matrix which encapsulates un-crosslinked PAAm-g-LBG copolymer along with ketoprofen. Further, during the treatment of such ionically cross-linked polyspheres with GA, there was formation of an acetal structure due to reaction between aldehyde groups present in GA and hydroxyl groups in SA and PAAm-g-LBG; thereby IPN containing SA and PAAm-g-LBG were formed.

3.4. SEM analysis

Fig. 1 depicts the SEM microphotographs of IPN polyspheres (L3: ionically cross-linked polyspheres) and L9 (ionic as well as covalent cross-linked polyspheres). The IPN polyspheres were found to be in spherical shape. It was noticed that ionically cross-linked L3 polyspheres were featured with the presence of rough and dense surface, while the L9 polyspheres formulated by means of ionic as well as covalent cross-linking carry even surface and minor surface foldings. Surface roughness was reduced to a greater extent due to the shrinkage at increased crosslinking resulting in formation of smoother surface [33].

3.5. Determination of size

Size of IPN polyspheres ranged between 857 ± 1.75 and 948 ± 2.01 µm as per the results summarized in Table 2. With the increase in amounts of PAAm-g-LBG and ketoprofen, size of IPN polyspheres was also increased. On the other hand, when the concentration of Ca^{2+} ions was enhanced, there was decrease in polysphere size. Also with the treatment of ionically crosslinked polyspheres with GA, the size of polyspheres was further reduced. At the time of crosslinking, rapid shrinkage was experienced by polymer matrix and hence smaller and stiffer IPN polyspheres might have formed at increased crosslink density [34].

3.6. Estimation of DEE

The results of DEE of IPN polyspheres are given in Table 2. The % of DEE was found to vary between 76.12% and 90.17%. The IPN

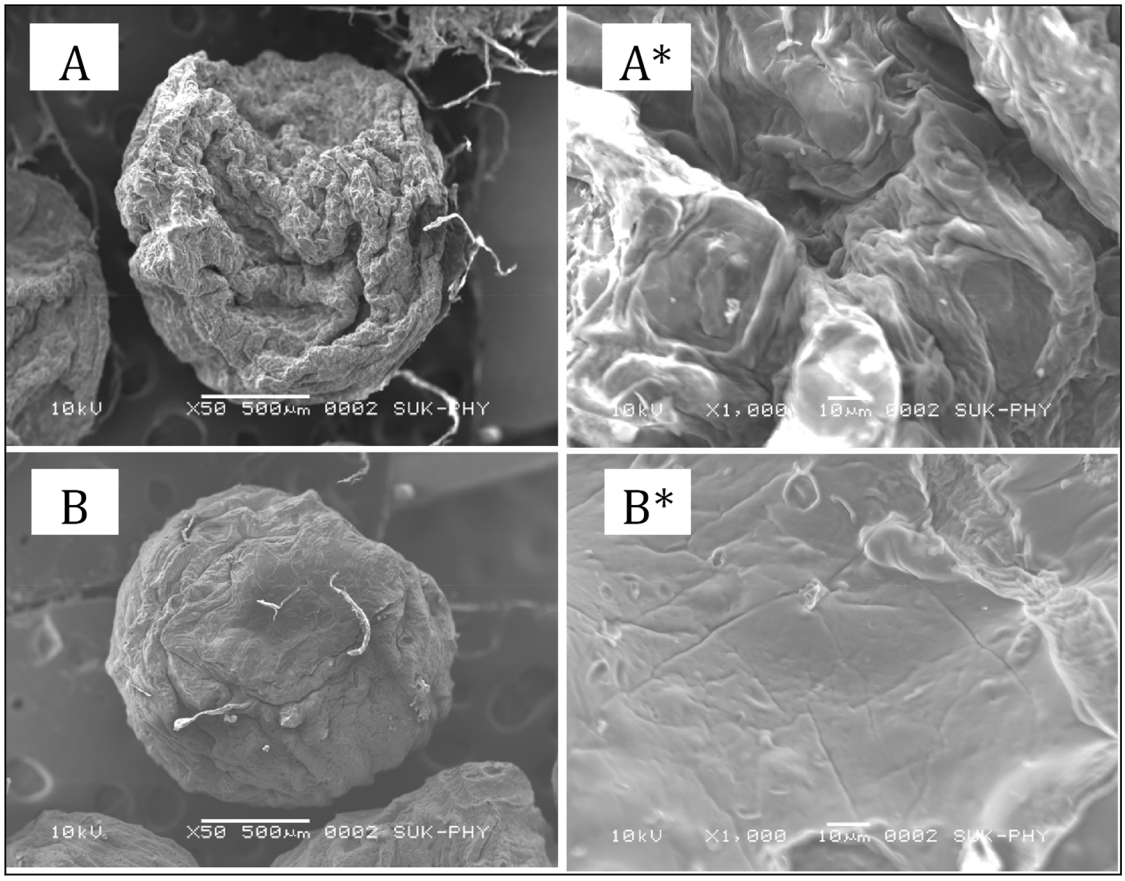

Fig. 1. SEM photographs of L3 IPN polyspheres (A) and L9 IPN polyspheres (B), A* and B* corresponds to their surface.

Table 2
Determination of size, DEE and drug release mechanism of IPN polyspheres.

IPN polyspheres	Average size (µm)	DEE (%)	Value of *n*	
			pH 1.2	pH 7.4
L1	915 ± 3.12	80.35 ± 0.79	0.98	0.76
L2	923 ± 2.12	82.73 ± 0.25	0.97	0.73
L3	931 ± 1.65	85.11 ± 0.76	0.96	0.71
L4	942 ± 1.32	88.69 ± 0.95	0.94	0.68
L5	948 ± 2.01	90.17 ± 1.54	0.92	0.67
L6	927 ± 1.14	86.90 ± 0.97	0.94	0.69
L7	901 ± 1.45	83.92 ± 0.48	0.97	0.71
L8	876 ± 1.75	81.54 ± 0.62	0.98	0.74
L9	861 ± 1.23	78.57 ± 0.45	0.99	0.79
L10	857 ± 1.75	76.12 ± 0.93	0.97	0.72

'n' indicates release exponent.

polyspheres formulated using lower concentration of Ca^{2+} ions exhibited lower DEE than those prepared by means of higher concentration of Ca^{2+} ions. This may be due to the saggy and leaky matrix formed as a result of inadequate crosslinking, thereby leakage of drug from IPN matrix. When Ca^{2+} ion concentration was increased, IPN matrix was rigid and drug leakage was reduced which might have resulted in higher DEE. On contrary, at the higher amount of PAAm-g-LBG copolymer, the DEE was increased to a little extent which could be due to increased size of polyspheres [35].

3.7. TG analysis

The TGA thermograms of SA, PAAm-g-LBG and L9 IPN polyspheres are portrayed in Fig. S4 under supplementary information. Weight loss of SA was in two phases; with 62.39% up to 400 °C and at the end of 600 °C a 80.11% weight loss was noticed. Initial weight loss was because of free and bound moisture evaporation from polymer, followed by weight loss at subsequent stages might be as a result of polymer degradation. PAAm-g-LBG encountered weight loss in one phase; with 57.76% till 400 °C and a final weight loss was found to be 66.65% at the end of 600 °C. But, L9 IPN polyspheres demonstrated weight loss in two stages; initial weight loss was found to be 55.67% till 400 °C and at 600 °C, a 60.69% of slow and constant weight loss was reported. In comparison with SA and PAAm-g-LBG, the residual mass of L9 IPN polyspheres was high; this demonstrates more thermal stability of IPN polyspheres, as SA and PAAm-g-LBG chains were closely associated by entanglement with crosslinking agent.

3.8. DSC analysis

DSC thermograms of plain ketoprofen, L9 IPN polyspheres without drug and L9 IPN polyspheres loaded with drug are cited in Fig. S5 under supplementary information. Endothermic peaks were exhibited at 188 °C and 186 °C by plain ketoprofen and drug loaded L9 IPN polyspheres, respectively. It was observed that endothermic peak was slightly shifted towards lower temperature with the drug loading; this was attributed by the loosely formed IPNs after drug loading. Based on the melting point of ketoprofen, an endothermic peak at 94 °C was observed in thermogram of plain ketoprofen, but the same peak was not present in drug-loaded L9 IPN polyspheres, thereby confirming existence of ketoprofen as amorphous dispersion in IPN matrix.

3.9. XRD analysis

X-ray diffractograms for plain ketoprofen, L9 polyspheres (without drug) and drug loaded L9 polyspheres are given at Fig. S6 under

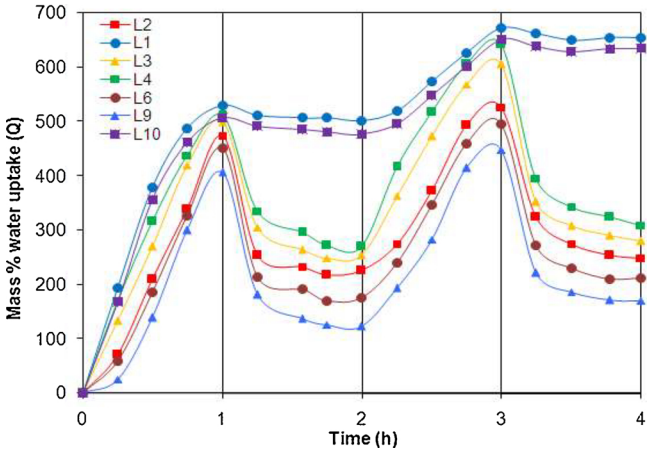

Fig. 2. Pulsatile swelling behavior of IPN polyspheres swollen in pH 1.2 solution and pH 7.4 solution.

supplementary information. Peaks between the 2θ of 5° and 25° recommended the existence of plain ketoprofen in crystalline state. But the drug related peaks were absent in case of L9 polyspheres loaded with drug, which confirmed the existence of ketoprofen as amorphous dispersion in the IPN matrix.

3.10. Pulsatile swelling study

Pulsatile swelling study results are presented in Fig. 2. The L1 polyspheres formulated using SA alone and L10 polyspheres prepared using native LBG with SA were non-responsive to changing pH; whereas a noteworthy response to pH was observed by IPN polyspheres formulated using combination of PAAm-g-LBG and SA. It was found that as a result of carboxyl groups present on PAAm-g-LBG, the IPN polyspheres experienced reversible swelling-shrinking behavior with the inter change of surrounding solution pH between 1.2 and 7.4. In alkaline medium, the carboxyl groups of IPN polyspheres might have undergone ionization thereby increasing osmotic pressure inside the matrix of IPN causing increased swelling [16].

3.11. In vitro drug release

Through *in-vitro* dissolution studies, the results of drug release characterization from IPN polyspheres are depicted in Fig. 3. Drug release from the L1 polyspheres formulated using SA alone was rapid; 40% drug was released in 1.2 pH buffer within 2 h and in pH 7.4 buffer, a 92% of drug was released within 6 h. While in case of L10 polyspheres formulated using native LBG with SA, drug release was also high. About 39% of drug was released within 2 h in pH 1.2 solution and about 90% of drug was released within 6 h in pH 7.4 phosphate buffer (Fig. 3B). As a result, the polyspheres formulated using native LBG and SA were found to be failed to retard release of drug in the acidic pH prevailing in stomach.

On the contrary, IPN polyspheres formulated using PAAm-g-LBG with SA (L3 to L9) have experienced pH-dependent drug release behavior due to $-COO^-$ functional groups present on PAAm-g-LBG backbone, where there was a controlled drug release till 12 h (Fig. 3A). Drug release was slow in 1.2 pH buffer in comparison with drug release in 7.4 pH buffer because of greater imbibement of IPN polyspheres in 7.4 pH buffer. It was inferred that amounts of PAAm-g-LBG and crosslinking agents affect the extent of drug release [36]. With an increase in Ca^{2+} ion concentration, the drug release was decreased. It was also noticed that the increase in concentration of PAAm-g-LBG attributed for drug release enhancement. Conversely, with increased initial drug loading, drug release was also increased; because of the fact that as initial drug loading increases, polymer ratio decreases and IPN matrix formed was found to be weak and resulted in increased drug release. IPN polyspheres, which were dually cross-linked using GA (L7 to L9), have exhibited slow drug release than that of ionically cross-linked IPN polyspheres (L2 to L6). Overall, there was a controlled drug release from dual cross-linked IPN polyspheres; in 1.2 pH buffer, there was about 10% drug release and about 88% of drug release occured in 7.4 pH buffer.

The empirical equation given below was applied so as to determine mechanism of drug transport from IPN polyspheres [37].

$$\frac{Mt}{M\infty} = Kt^n \tag{3}$$

M_t represents release of drug at time t; M_∞ defines total amount of drug loaded, n values demonstrate mechanism of transport. With values of n ranging between 0.92 and 0.99 in pH 1.2 solution, the polyspheres were found to follow case II transport mechanism and n values ranging from 0.67 to 0.79 in pH 7.4 solution, the polyspheres followed anomalous transport mechanism (Table 3). Value of n increased as concentration of crosslinking agents was increased.

3.12. In-vivo pharmacokinetics

Table 3 summarizes the data obtained by *in-vivo* pharmacokinetic assessment. The T_{max} values of plain ketoprofen and L9 IPN polyspheres were 1 h and 6 h respectively, indicating faster absorption of plain ketoprofen after oral administration when compared to drug loaded L9 IPN polyspheres. The C_{max} value of L9 IPN polyspheres was lower when compared with plain ketoprofen, which might be because of gradual ketoprofen absorption from IPN polyspheres. The elimination half-life ($t_{1/2}$) pertaining to IPN polyspheres was higher (4.24 h) than plain ketoprofen (4.05 h), demonstrating gradual ketoprofen elimination from IPN polyspheres. The L9 IPN polyspheres along with plain ketoprofen exhibited elimination rate constant values (Ke) of 0.163 ± 0.018 and $0.171 \pm 0.020\ h^{-1}$, respectively. IPN polyspheres presented greater values of AUC when compared with plain ketoprofen, thereby suggesting the preferred bioavailability of ketoprofen because of controlled drug release from IPN polyspheres up to ≈ 10 h (Fig. S7, as shown under supplementary information).

There was an observation that plain ketoprofen exhibited immediate raise in plasma concentration of drug after oral administration followed by decrease after 1.5–2 h; whereas, the IPN polyspheres exhibited low plasma concentration of drug till 2nd h in comparison with plain ketoprofen. The above observation showed faster absorption of plain ketoprofen, quick presence in plasma within 2 h followed by faster elimination, which might be because of shorter half-life. While with pH-sensitive IPN polyspheres, there was lesser release of drug in acidic pH of stomach as a result of unionization of polyspheres. With polyspheres movement down the stomach into intestine *i.e.*, alkaline environment, ionization of polyspheres occurs leading to maximum swelling followed by drug release. The pharmacokinetics parameters of L9 IPN polyspheres seemed to be predominantly different when compared with pristine ketoprofen ($p < 0.05$).

3.13. Anti-inflammatory activity

The outcome of anti-inflammatory action is shown in Fig. 4. Plain ketoprofen exhibited 88.0% edema suppression of paw in less than 2 h, followed by decrease in percentage edema inhibition and finally it reached 7.21% by the end of 12th h. In case of L9 IPN polyspheres which were pH sensitive, the percent edema inhibition was initially low *i.e.*, 6.45% till 2 h and slowly it enhanced to 95.36% by end of 8th h and then reduced to 75.46% by the end of 12th h. This demonstrates the maximal activity of plain ketoprofen in less than 2 h followed by elimination phase; whereas from pH-sensitive IPN polyspheres, there was less release of drug in gastric pH but maximum amount of drug release occurred in intestinal environment.

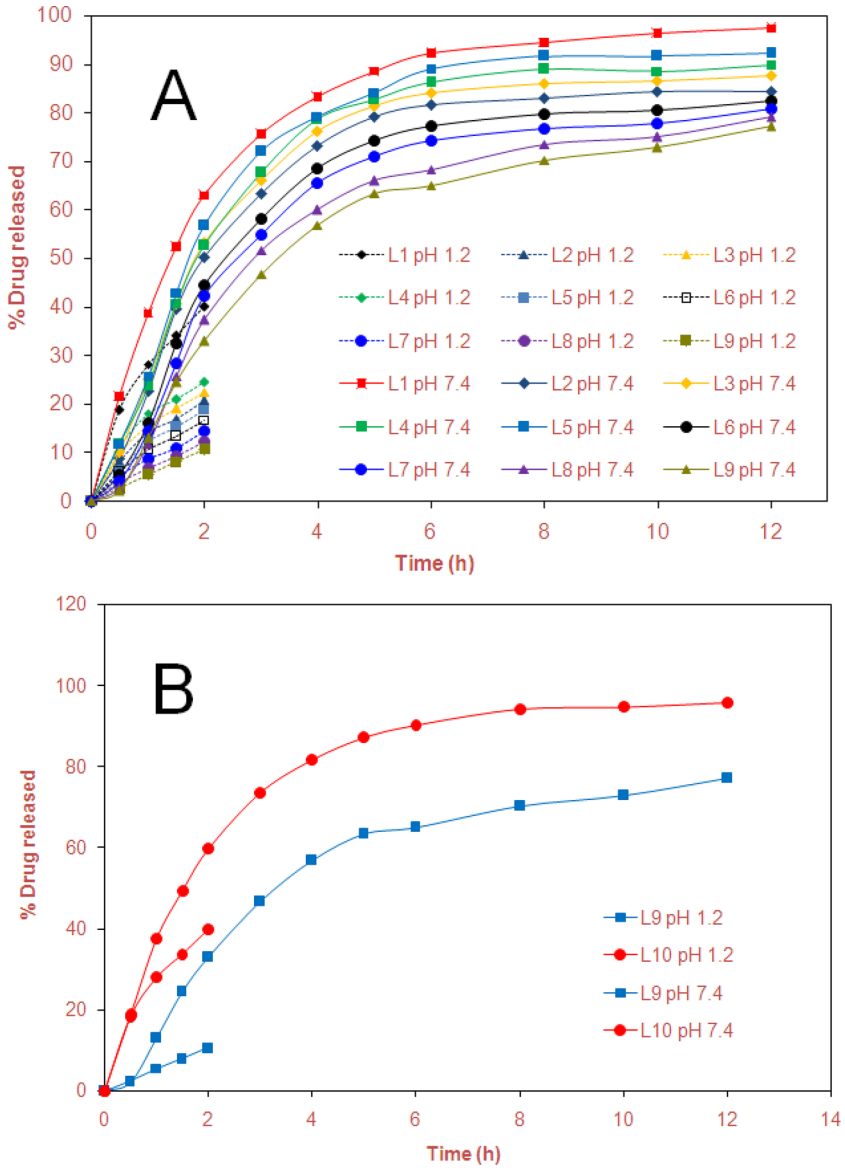

Fig. 3. *In vitro* drug release profiles of IPN polyspheres prepared using PAAm-g-LBG with SA (A) and native LBG with SA (B).

Table 3
Pharmacokinetic parameters for ketoprofen (PD) and L9 IPN polyspheres.

Pharmacokinetic parameters	PD	L9 IPN polyspheres
C_{max} (μg/mL)	4.48 ± 0.29	4.39 ± 0.26
T_{max} (h)	1.00 ± 0.00	6.00 ± 0.00
$AUC_{0-\infty}$ (μg.h/mL)	33.90 ± 3.28	46.20 ± 3.98
AUC_{0-t} (μg.h/mL)	33.50 ± 3.31	45.00 ± 2.06
MRT (h)	5.90 ± 0.38	8.70 ± 0.56
$T_{1/2}$ (h)	4.05 ± 0.26	4.24 ± 0.31
K_e (1/h)	0.171 ± 0.020	0.163 ± 0.018

Results are mean ± SD, n = 6. AUC, area under the curve; Cmax, maximum plasma concentration; Ke, elimination rate constant; Tmax, time for maximum plasma concentration; t1/2, half life·.

3.14. Stomach histopathology

Fig. 5 and Table 4 shows an outcome of stomach histopathological study carried out on control group rats (administered with vehicle), standard group rats (administered with plain ketoprofen) and test group rats (administered with L9 IPN polyspheres). No signs of ulcer/hemorrhages were found in control group rats (A) and normal mucosal layer intactness and unaltered surface epithelium was seen. For the rats where plain ketoprofen was administered (B), ulcers measuring 1.97 ± 0.72 mm along with hemorrhages were found. Prominent mucosal erosion with congestion, oedema and perforations was observed. In addition, surface epithelium seemed to be irregular and gastric glands were affected. On the other hand, in test group rats (C), small ulcers of about 0.11 ± 0.14 mm with no perforation, congestion, hemorrhages and necrosis were noticed. But there was a slight oedema and mucosal erosion; however the gastric glands seemed to be normal and intact, indicating ketoprofen associated adverse events such as ulcers, hemorrhage and mucosal erosions were decreased after its encapsulation into pH-sensitive IPN polyspheres.

4. Conclusions

The successful development of pH-sensitive IPN polyspheres was achieved with PAAm-g-LBG along with SA for targeting ketoprofen to small intestine. Synthesis of pH-sensitive PAAm-g-LBG copolymer was carried out by means of microwave irradiation method followed by alkaline hydrolysis. PAAm-g-LBG copolymer was characterized by ^{1}H-NMR, FTIR and elemental analysis. IPN polyspheres of PAAm-g-LBG in

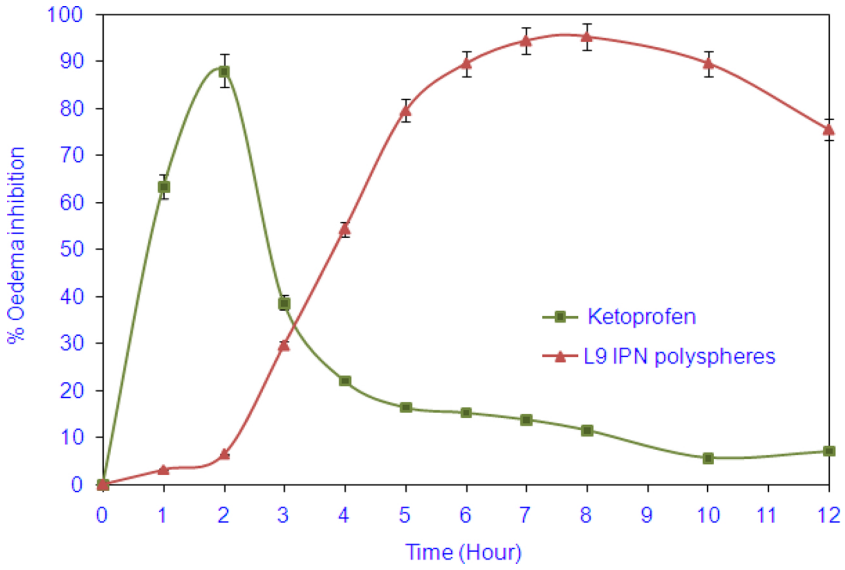

Fig. 4. Screening of anti-inflammatory activity of pristine ketoprofen (A) and pH-sensitive L9 IPN polyspheres (B) in carrageenan-induced rat paw edema.

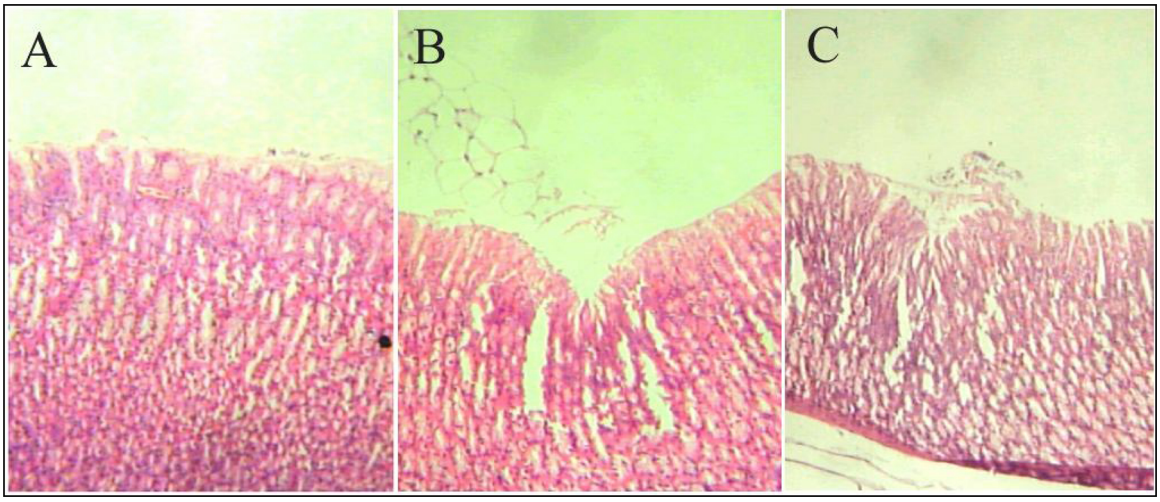

Fig. 5. Stomach histopathology of control rats (A), rats treated pristine ketoprofen (B) and rats treated with L9 IPN polyspheres (C) (Haematoxylin-Eosin, 100X).

Table 4
Stomach histopathology of rats treated with vehicle, pristine ketoprofen and L9 IPN polyspheres.

Sl. No	Parameters	Rat groups		
		Control group rats	Rats treated with pristine ketoprofen	Rats treated with L9 IPN polyspheres
01	Ulcer number	0	06 ± 0.91	0.7 ± 0.76
02	Ulcer size (mm)	0	1.97 ± 0.72	0.11 ± 0.14
03	Perforation	0	2	0
04	Hemorrhage	0	3	0
05	Congestion	0	3	0
06	Oedema	0	3	1
07	Necrosis	0	3	0
08	Erosion of gastric mucosa	0	4	1

Values are mean ± S.D. of five animals in each group. Score: 0: Nil; 1: Mild; 2: appreciable; 3: severe; 4: very severe.

combination with SA were prepared by means of dual crosslinking method. The optimized IPN polyspheres (L9) exhibited pH-depended reversible swelling and shrinking with the alteration in pH of surrounding media. IPN polyspheres released ketoprofen at a higher rate (about 90%) in phosphate buffer of pH 7.4 when compared with drug released in acidic buffer of pH 1.2 (about 10%). The *in-vivo* pharmacokinetics, stomach histopathology and anti-inflammatory activity studies demonstrated pH-sensitivity of IPN polyspheres where ketoprofen was successfully targeted to small intestine, thereby side effects associated with ketoprofen were diminished.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.colsurfb.2019.04.060.

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