Research Article

Genipin-Crosslinked Gelatin-Based Emulgels: an Insight into the Thermal, Mechanical, and Electrical Studies

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Abstract. The present study discusses about the preparation and characterization (thermal, mechanical, and electrical) of the genipin-crosslinked gelatin emulgels. Emulgels have gained importance in recent years due to their improved stability than emulsions and ability to control the drug release. Mustard oil was used as the representative oil. A decrease in the enthalpy and entropy of the formulations was observed with the increase in the oil fraction. The mechanical studies suggested formation of softer emulgels as the oil fraction was increased. As the proportion of the oil fraction was increased in the emulgels, there was a corresponding increase in the impedance. The drug release properties from the emulgels were also studied. Ciprofloxacin was used as the model antimicrobial drug. The drug release was higher from the emulgels whose electrical conductivity was higher.

KEY WORDS: drug delivery; emulgels; gelatin; genipin.

INTRODUCTION

Hydrogels may be defined as the polymeric constructs with imbibed water. They have been extensively studied for various biomedical applications including tissue engineering and drug delivery. Hydrogels have been synthesized using either synthetic or natural polymers (1). The use of natural polymers is generally preferred as compared with the synthetic polymers. This is due to the easy availability and wide range of chemistries available in the natural polymers. Various natural polymers (e.g., gelatin, chitosan, alginate, celluloses, and collagen) have been used for the designing of the hydrogels for controlled applications (2–6). Gelatin, a protein-based biopolymer derived from animal collagen, is one such natural polymer which has been explored extensively to develop controlled delivery matrices. Gelatin is one of the highly preferred natural polymers. This can be explained by the biocompatible nature of gelatin (6–9). The main problem associated with the gelatin construct is the weak mechanical property of the gel-

atin construct near or above body temperature which results in the alteration in the drug release properties from the gelatin construct. This may be overcome by crosslinking the gelatin construct using a suitable crosslinker (e.g., glutaraldehyde and genipin). Genipin is a naturally occurring crosslinker obtained from the fruits of *Gardenia jasminoides*. It has been gaining importance in biomedical industries due to its far less cytotoxic nature as compared with glutaraldehyde (10,11).

In recent years, emulgel has emerged as a promising delivery vehicle. The word “emulgel” has been derived from the words emulsion and gels. Emulsions are biphasic liquid formulations which are categorized either as water-in-oil or oil-in-water types. They have been conventionally used for drug delivery applications. Inherently, emulsions are thermodynamically unstable in nature. On the other hand, gels are semisolid formulations with improved thermodynamic stability. Gels are preferred for mucoadhesive drug delivery. The mucoadhesive property helps achieving higher bioavailability of drugs at the site of action. Emulgel combines the beneficial properties of both emulsion and gels. Emulgel may be visualized as an emulsion whose continuous phase has been converted into a gel (12,13). Due to this, the thermodynamic instability of the emulsions can be improved without compromising with the controlled delivery of the drugs. Additionally, the mucoadhesive property of the formulations is an added advantage. But in our previous study, we have found that the mucoadhesive property is dependent on the composition of the emulgel (12).

Mustard oil has been traditionally extensively used in southern Asian countries (India, Pakistan, and Bangladesh) for skin massages of infants and adults (14–16). Apart from this, mustard oil containing emulsions have been developed for the topical

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ocular delivery of non-steroidal anti-inflammatory drugs (NSAIDs) *viz.*, diclofenac (17). In recent years, mustard oil has been used as an adjuvant for the development of novel new nutritive-immune-enhancing delivery system (18,19). Taking inspiration from the above studies, mustard oil was used as the representative oil in this study.

Previously, genipin-crosslinked gelatin emulgels have been reported by our group for the delivery of indomethacin (20). The crosslinking of the emulgels were carried out by diffusion of genipin into the uncrosslinked emulgels. This promoted crosslinking of the gelatin matrix in a graded manner thereby resulting in the formation of matrices with heterogeneous properties across the thickness. The crosslinking density of the upper layer of the matrix was higher, whereas the crosslinking density was lower in the lower part of the matrix. This type of material is useful as wound dressings. In the present study, gelatin and mustard oil-based emulgel matrices were developed and characterized thoroughly as controlled delivery vehicles. The pH of the developed formulations was in the range of 5.5 and 7.0, which is near to the physiological pH (21). Due to this reason, it was expected that the formulations will be non-irritant. Genipin-crosslinked gelatin emulgels were prepared by *in situ* crosslinking of the gelatin molecules. In the recent past, emulgels have been developed as controlled delivery matrices for topical, transdermal, and vaginal drug delivery applications (22,23). The formulations were prepared using varied proportions of gelatin solution and mustard oil. The proportion of genipin was kept constant. This resulted in the variation in the gelatin (polymer)/genipin ratio. The prepared formulations were thoroughly characterized for understanding the thermal, mechanical, electrical, and drug (ciprofloxacin) release properties of the prepared emulgels.

MATERIALS AND METHODS

Materials

Tween 80 (polyxyethylene sorbitan monooleate), gelatin, and glycine were procured from Himedia, Mumbai, India. Ciprofloxacin (CF) was procured from Fluka Biochemica, China. Genipin was procured from Challenge Bioproduct Company Limited, China. Edible-grade refined mustard oil (MO; Gokul refoils and solvent Ltd., Gujrat, India) was purchased from the local market. Double distilled water was used throughout the study.

Preparation of Emulgels

The emulgels were prepared as per the method reported earlier with slight modifications in the procedure (12). In short, 20% (w/w) gelatin solution was prepared by dissolving 20 g of gelatin in 80 g of water, kept on stirring at 50°C (GS). To this clear homogeneous solution, mustard oil (50°C) was added to the gelatin solution and homogenized using overhead stirrer at 800 rpm for 15 min to form primary emulsion; 0.1 g of genipin was added to the primary emulsion and further homogenized for 30 s. The emulsion was subsequently poured into petri plates and incubated at 40°C for 30 min to promote crosslinking of the gelatin matrix. Excess genipin was neutralized by 1% (w/w) glycine solution (24). The crosslinked gels were washed thoroughly with water and stored under

refrigerated conditions (5°C). Ciprofloxacin-loaded emulgels were prepared in a similar manner so as to have 1% (w/w) ciprofloxacin in the formulations. Ciprofloxacin was dissolved in mustard oil for the preparation of the emulgels. The gels were stored under refrigerated conditions for further analysis.

The microstructures of the emulgels were analyzed under scanning electron microscope (SEM; JSM-6390, JEOL, Japan) after converting them into xerogels. The xerogels were prepared by drying the gel samples for 2 weeks under vacuum. The xerogels were with platinum prior to visualization under the SEM (25). The infrared spectroscopy of the emulgels and drug-loaded emulgels were recorded in the range of 400–4000 cm using Bruker ALPHA-E FTIR spectrophotometer (USA) (26).

Thermal Studies

The emulgels were analyzed in the temperature range of 20–150°C at 2°C/min scan rate under N₂ environment using differential scanning calorimeter (DSC-200 F3 Maia, Netzsch, Germany) (27).

Mechanical Testing

Mechanical properties of the emulgels were studied using texture analyzer (Stable Microsystems, TA-HDplus, UK). The stress relaxation and the compressive properties of the formulations were determined (28). The tests were conducted at room temperature (25°C). Table S1 summarizes the instrumental parameters for conducting the tests.

Electrochemical Impedance Spectroscopy

The electrical properties of the emulgels were studied using an impedance analyzer (PSM 1735, Numetrix, UK) in the frequency range of 0.1 Hz to 1.0 MHz (AC voltage of 100 mV). The tests were conducted at room temperature (29,30).

In Vitro Drug Release Studies

The drug dissolution tests (quantitative) of the emulgels were conducted in a single-basket dissolution apparatus for 8 h. The drug containing emulgels (~1.5 g) were put into the dissolution basket containing 900 ml of water (37°C). Three milliliters of samples were withdrawn at regular intervals of time (15 min for the first hour and 30 min for the next 7 h) and were replaced with fresh dissolution medium. The samples were analyzed at 271 nm using UV–visible spectrophotometer.

Drug release kinetics was studied using two different mathematical models *viz.*, Higuchian model and Korsmeyer-Peppas (KP) model (31).

$$\frac{M_t}{M_o} = k_H \cdot t^n \quad (1)$$

$$\frac{M_t}{M_o} = k_p \cdot t^n \quad (2)$$

Where, M_t is the amount of drug released at time t , M_o is the amount of drug loaded, k_H is the Higuchian rate constant, k_p is the Peppas rate constant, and n is the Fickian value.

Fig. 1. cEHs of different compositions: **a** cG1, **b** cG2, **c** cG3, **d** cG4, and **e** cG5

Statistical significance on the differences in the drug delivery profiles was calculated by one-way ANOVA using Microsoft Excel 2007 software.

Antimicrobial Studies

The qualitative drug dissolution test was done to understand the ability of the formulations to release the drug in active form. Gram-positive bacterium *Bacillus subtilis* MTCC 121 was used for the study. One milliliter of cell suspension (containing 10^6 to 10^7 cfu/ml) in nutrient broth was spread over the solid nutrient agar medium. Bores of 9-mm diameter were made into the agar plates using a stainless steel borer. Emulgels were put into the bores. Ciprofloxacin powder served as the positive control. Powdered antimicrobial agents have been previously reported to show antimicrobial activity (32). Blank emulgels served as the negative control. The petri plates were incubated at 37°C for 24 h. The zone of inhibition of the bacteria was measured after 24 h (33).

RESULTS AND DISCUSSION

Preparation of Emulgels

The formation of the stable emulgels was dependent on the proportion of the gelatin solution and mustard oil present in the formulation. Structurally, stable emulgels were formed when the gelatin solution proportion was either equal to or higher than the proportion of the mustard oil (1:0, 7:1, 3:1, 1.67:1, 1:1) (Fig. 1; Table I). With the further increase in mustard oil proportion (gelatin solution/mustard oil ratio=1:1.67, 1:3, 1:7), phase separation of the formulation was observed. The microstructures of the emulgels were visualized under SEM as xerogels (Fig. 2a–c). A xerogel is a polymeric matrix (hydrogel) devoid of the aqueous phase. The water was removed from the gels by drying the gels under vacuum at 40°C. cG1, cG3, and cG5 gels were chosen as the representative samples. The micrographs of the xerogel showed the presence of globular structures. As the proportion of the oil was increased, there was a corresponding increase in the globular structure and the surface roughness of the xerogels.

Table I. Composition of the Prepared Gels

Samples	Mass of 20% (w/w) gelatin solution (g)	Mass of MO (g)	Genipin (g)	Gelatin: genipin	Ciprofloxacin (g)	Result
cG1	20.0	0.0	0.1	40:1	–	Gel formed
cG2	17.5	2.5	0.1	35:1	–	Gel formed
cG3	15.0	5.0	0.1	30:1	–	Gel formed
cG4	12.5	7.5	0.1	25:1	–	Gel formed
cG5	10.0	10.0	0.1	20:1	–	Gel formed
cG6	7.5	12.5	0.1	15:1	–	Phase separation
cG7	5.0	15.0	0.1	10:1	–	Phase separation
cG8	2.5	17.5	0.1	5:1	–	Phase separation
cG1D	19.8	0.0	0.1	39.8:1	0.2	Gel formed
cG3D	14.8	5.0	0.1	29.6:1	0.2	Gel formed
cG5D	9.8	10.0	0.1	19.6:1	0.2	Gel formed

MO mustard oil

Fig. 2. The micrographs of the xerogels under SEM: **a** cG1, **b** cG3, and **c** cG5; IR spectroscopy of the **d** raw materials and **e** blank and drug-loaded gels

Ciprofloxacin showed peaks at 3378 and 1178 cm⁻¹ (Fig. 2d). These peaks were due to free -OH and C=O stretching vibration of the carboxylic acid groups. The peaks at 3528 and 1624 cm⁻¹ can be associated with the stretching and bending vibration of amines, respectively (34). Genipin showed peak at 1414 cm⁻¹ corresponding to the ring stretching in genipin (Fig. 2d). (35). The characteristic peaks of gelatin were conserved in the spectra of the emulgels. Gelatin gel showed peaks at 1638, 1558, and 1244 cm⁻¹ due to the presence of the amide bonds (Fig. 2e) (36). The peaks (1244 to 1241 and 1163 to 1161 cm⁻¹) were slightly shifted towards the lower wave numbers in the emulgels. This was due to the crosslinking of gelatin with genipin (37). The wide absorption band at 3319 cm⁻¹ was due to the hydrogen bonding due to the presence of O-H and N-H groups of gelatin in the gelatin gel. This peak was conserved in emulgels (36). The results suggested that the interconnected triple helices of gelatin polypeptide chains were conserved even after crosslinking. Increase in the oil proportion in the emulgels resulted in the decrease in the intensity of the peak. This can be explained by the presence of lower fraction of gelatin in the emulgels with higher proportions of oil. This resulted in the increased crosslinking density of the gelatin matrices, which in turn, resulted in the reduction in the free amino groups available for hydrogen bonding.

Thermal Studies

Thermal properties of the emulgel components (gelatin, genipin) and the representative emulgels (cG1, cG3, and cG5) were analyzed in depth (Fig. 3). Gelatin did not show any distinct endotherm over the experimental temperature range. On the other hand, genipin showed a sharp endotherm at 120°C. Presence of sharp peak indicates that the crosslinker is in its pure form. The peak associated with the crosslinker

Fig. 3. The DSC curves of cEHs

Table II. Change in Enthalpy and Entropy of the Emulgels

Samples	ΔH_m (J/g)*	ΔS_m (J/g K) ^a
cG1	-1072.0 $\pm$ 3.39	-436.49 $\pm$ 4.48
cG3	-459.5 $\pm$ 4.55	-173.97 $\pm$ 4.23
cG5	-257.4 $\pm$ 3.87	-96.84 $\pm$ 2.65
Genipin	-119.5 $\pm$ 0.21	-19.23 $\pm$ 0.35

^aNegative sign is due to endothermic convention

was not found in the thermal profiles of the emulgels. Since genipin is completely dissolved in the gelatin solution and is involved in crosslinking of the gelatin molecules by interacting with the primary amines, the endotherm corresponding to the genipin was not observed (38). The emulgels showed a predominant broad endothermic peak. This can be accounted to the evaporation of water present in the emulgels. The endothermic peak was observed at 98.88, 106.14, and 106.19°C for cG1, cG3, and cG5, respectively. The change in enthalpy (ΔH_m) and change in entropy (ΔS_m) values associated with the endothermic peaks were calculated (Table II). The results suggested that with the increase in the oil fraction, there was a corresponding decrease in the ΔH_m and ΔS_m values. This suggested that less energy was needed to evaporate the water molecules from the emulgels. This can be explained by the reduction in the hydrogen bonding in the emulgels with higher proportions of oil (as evident from the FTIR studies) (39).

Mechanical Analysis

Stress relaxation helps understanding the ability of the formulations to absorb stress due to the polymeric molecular rearrangements (Fig. 4; Table S2). There was a decrease in the force required to penetrate the target distance (F_0) as the proportion of the oil was increased (Fig. 4a). This suggested that the firmness of the emulgels was reduced with the increase in the oil proportion. The firmness of the gels was also confirmed by calculating the D_{25} (distance moved by the probe to attain a 25 $\times$ g force). It was found that the distance moved by the probe was highest in cG5 followed by cG3 and cG1, respectively (Fig. 4b). The percentage of relaxation of the formulations was calculated using F_0 and the residual force (F_{60}) at the end of the experiment. Work on relaxation is often regarded as “work softening (WS)” (40). The WS of the formulations divulges information about the ability of the formulations to deform when a stress (force) is applied. A decrease in the work on relaxation and the percentage of relaxation was observed with the increase in the oil content. The results suggested that the formulations containing higher proportions of oil were softened to a greater extent when stress was applied.

The hysteresis curves obtained during the stress relaxation studies of the emulgels showed that a closed hysteresis loop was obtained in cG1 and cG3. The closed loop was not observed in cG5. A closed hysteresis loop suggested total reconciliation to the initial microstructure as the stress was released. Hysteresis loop area provides information about the

Fig. 4. Stress relaxation of cEHs in terms of **a** force vs time curves, **b** force vs distance curves, **c** modified Peleg's analysis curves, and **d** the compression studies of cEHs

Table III. Viscoelastic Parameters of the Emulgels

Samples	F_0 (g)	F_r (g)	k_1	k_2 (10^{-5})	S^*	F^*	% relaxation	Maximum load (kg)
cG1	303.15	236.46	0.11	9.0	0.86	0.783	78.099	11.33 ± 2.12
cG3	163.32	103.76	0.07	8.0	0.85	0.734	73.537	4.68 ± 2.24
cG5	35.98	10.12	0.077	8.0	0.84	0.735	74.099	2.53 ± 1.01

amount of work done to regain the initial structure (Table S3) (41). With the increase in the oil proportion, there was a decrease in hysteresis loop area. This suggested a decrease in the elastic behavior of the emulgels as the proportion of the oil was increased. Hence, a less amount of work was needed to regain the initial structure in cG1 and cG3 (42).

The viscoelastic nature of the emulgels was further confirmed by fitting the stress relaxation profiles as per the modified Peleg's equation (43).

$$\frac{(F_0 - F(t))t}{F_0} = k_1 + k_2 t \quad (3)$$

where, F_0 is the maximum force attained after loading; k_1 and k_2 represent the initial rate and extent of the relaxation, respectively.

The normalized stress relaxation data was found to be fitting ($R^2 > 0.995$) in the modified Peleg's equation (Fig. 4c). The fitting parameters (k_1 and k_2) were tabulated in Table III. Even though the initial rate of relaxation (k_1) was lower in emulgels when the proportion of the oil was higher, the extent of relaxation (k_2) was almost uniform for all the formulations. Normalized stress relaxation curve (F^*) and the area under the normalized stress relaxation curve (S^*) was calculated as

$$F^* = \frac{F_r}{F_0} \quad (4)$$

$$S = \int_a^b F(t) \cdot dt \quad (5)$$

$$S_0 = \left(\frac{S}{b-a} \right) a \quad (6)$$

$$S^* = \frac{S}{S_0} \quad (7)$$

where, a and b are the lower and upper limits of the time, respectively.

Fig. 5. Impedance spectroscopy of the cEHs: **a** Nyquist plot, **b** frequency dependent ϵ' , **c** $\tan \delta$, and **d** σ_{ac}

Table IV. DC Conductivity (σ_0) of the Formulations

Samples	DC conductivity(σ_0) ($\times 10^{-3}$) (S/cm)
cG1	1.60 $\pm$ 2.21
cG3	1.05 $\pm$ 2.28
cG5	0.51 $\pm$ 3.32

The compressive strength of the emulgels was determined using emulgels prepared as cylinders (height, 20 mm; diameter, 15 mm). The maximum load during compression was observed in cG1 followed by cG3 and cG5 (Fig. 4d). This suggested higher load bearing capacity of the emulgels with lower proportions of oil and may be explained by the higher elastic component in the emulgels with lower proportions of oil (evident from the stress relaxation study).

Electrochemical Impedance Spectroscopy

The Nyquist plots (Z'' vs. Z') of the emulgels showed formation of two semicircles which were not complete (limitation of the analyzer used) (Fig. 5a). The intersection of the plot with the x-axis provides information about the bulk resistance of the formulations. The bulk resistance was lowest in followed by cG3 and cG5, respectively. This can be explained by the presence of higher fraction of aqueous phase in cG1 followed by cG3 and cG5, respectively (45). All the formulations showed higher impedance at lower frequencies which was lowered and reached a plateau at higher frequencies. This observation can be explained by the polarization of the electrode-formulation interface at lower frequencies. At higher frequencies, the polarization effect was minimized

due to the rapid periodic reversal of the a.c. field (46). The order of the impedance was cG1 < cG3 < cG5. The dielectric constant (ϵ') profile of the formulations was similar to that of the impedance profile (Fig. 5b). An increase in the proportion of the oil in the emulgels resulted in the decrease in the dielectric constant (ϵ'). The tangent loss ($\tan \delta$) profile provides information about the polymer relaxation. All the formulations contained gelatin as the polymer and explains the occurrence of the $\tan \delta$ peak at the same frequency (Fig. 5c) (47). Since the crosslinking density was varied in the formulations, the amount of loss was higher. When the proportion of the oil was lowest in cG1, the $\tan \delta$ peak was lowest indicating highest elastic properties. An increase in the oil proportion resulted in increase in the $\tan \delta$ peak. The loss in cG3 and cG5 were insignificant suggesting similar viscoelastic properties (47,48). The conductivities of the emulgels suggested frequency independent behavior at lower frequencies which got dispersed at higher frequencies (Fig. 5d) (49). The conductivity of cG1 was highest followed by cG3 and cG5, respectively. The results are in accordance with the bulk resistance values which showed bulk resistance in the order of cG1 < cG3 < cG5. As a matter of fact, the conductivity of the formulations is inversely proportional to the bulk resistance (50). The d.c. conductivity of the formulations was calculated by the linear extrapolation of the high frequency a.c. conductivity to d.c. frequency. The order of the d.c. conductivity was in the order of cG1 > cG3 > cG5 (Table IV).

In Vitro Drug Release Studies

The quantitative drug release study was carried out in a single-basket USP dissolution apparatus. The rate of drug release was found to be dependent on the composition of

Fig. 6. *In vitro* release studies: **a** CPDR profiles of the gels; fitting of the release profile of the gels **b** Higuchi, **c** KP kinetics, and **d-f** antimicrobial studies of the gels against *B. subtilis*

the emulgels. A higher amount of drug release was found in cG1D, followed by cG3D and cG5D, respectively (Fig. 6a). This may be explained by the partition coefficient of the drug, which states that the solute distributes itself amongst the two immiscible liquids in a definite concentration ratio (51–53). Since the aqueous fraction was highest in cG1D, the partitioning of the drug into the aqueous phase was highest in cG1D and hence the drug release. Partitioning of the drug into the aqueous phase was limited with the increase in the oil fraction of the emulgels. This resulted in the lower cumulative percentage of drug release (CPDR) from cG5D and cG3D, respectively, as compared with cG1D. The drug release profiles of cG1D and cG3D showed a near-linear drug release profile. On the other hand, the release profile of cG5D initially followed a near-linear profile for 4 h. Thereafter, the release profile reached plateau phase. The differences in CPDR value of cG5 and cG1/cG3 was found to be statistically significant ($p < 0.05$) (Fig. S1). On the other hand, the difference in the CPDR value between cG1 and cG3 was statistically insignificant ($p > 0.05$).

Drug release behavior from the emulgels was predicted by fitting Higuchian and KP models. Drug release from the emulgels showed good fitting towards both Higuchian and KP kinetic models ($R^2 > 0.95$). Higuchian kinetics happens when the delivery vehicles are matrix type (54). This suggested that the developed emulgels were matrix type. The Fickian or n value (slope of the KP model) was found to be in between 0.45 and 0.85. This suggested that the predominant mechanism for the release of the drug from the emulgels was non-Fickian diffusion mediated (55,56). This type of drug diffusion mechanism is typical for polymeric systems, in which the drug transportation is not controlled by either concentration gradient or macromolecular relaxation rate. But, the drug diffusion is controlled by the superposition of both diffusion and relaxation phenomena (57).

Antimicrobial Studies

The qualitative drug release of the drug from the formulations was determined by checking the antimicrobial efficiency of the ciprofloxacin-loaded emulgels against *B. subtilis* (Fig. 6d–f). A clear zone of inhibition was found against the pure drug (CF) and drug-loaded emulgels but not against blank emulgels (emulgels without drug). Presence of zone of inhibition indicated that ciprofloxacin was present in its active form in the emulgels. Zone of inhibition was found to be highest in cG1D, followed by cG3D and cG5D, respectively. Differences in the zone of inhibitions can be attributed to the amount of drug released from the emulgels.

CONCLUSION

The study reports the preparation and characterization of gelatin-based emulgels. The emulgels were crosslinked with genipin, a natural crosslinker. The properties of the emulgels were found to be dependent on the composition. An increase in the oil proportion resulted in the reduction in the hydrogen bonding amongst the gelatin and the water molecules. This resulted in the lower enthalpy and entropy associated with the evaporation of water molecules from the emulgels. Increase in the oil proportions resulted in the formation of softer emulgels

and reduction in the conductivity. The release of the drug was higher from the emulgels with lower fractions of oil and was found to be diffusion mediated. The rate of release of the drugs could be modulated by altering the composition of the emulgels.

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