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Synthesis and Characterization of an Enzyme Mediated *in situ Forming* Hydrogel Based on Gum Tragacanth for Biomedical Applications

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Background: The excellent biocompatibility, biodegradability and biological properties of the hydrogels, fabricated using natural polymers, especially polysaccharides, are very advantageous for biomedical applications. Gum tragacanth (GT) is a heterogeneous highly branched anionic polysaccharide, which has been used extensively in food and pharmaceutical industries. Despite, its desirable properties, the potential biomedical applications of this natural gum have not been fully explored. In this study, an enzyme catalyzed *in situ* forming hydrogel, based on Iranian gum tragacanth (exudate of *Astragalus fluccosus*) was prepared and characterized for biomedical applications.
Objectives: The main objective of the present study was to explore the feasibility of using tragacanth natural gum as a base for *in situ*-forming hydrogels in biomedical applications.
Materials and Methods: First, tyramine (TA) was conjugated to the water-soluble part of GT (TGA) using aqueous-phase carbodiimide activation chemistry. Next, *in situ* forming hydrogel was prepared via an enzyme catalyzed coupling reaction in the presence of horseradish peroxidase (HRP) and H_2O_2. Gelation time, swelling/degradation behavior and mechanical properties of the hydrogel and cell viability of the encapsulated cells within these hydrogels were investigated.
Results: The gelation time of the hydrogel was less than 30 seconds, which is very desirable for clinical applications. At concentrations ≤ 0.1% (w/v), both GT and TA-TGA showed no toxicity towards human mesenchymal stem cells (hMSCs) and Caco-2 cells. More than 90% of the encapsulated hMSCs in the hydrogels, which were prepared at H_2O_2 concentrations of less than 15.0 mM, remained viable after 2 hours of incubation.
Conclusions: The TA-TGA conjugate can be gelled enzymatically in the presence of HRP and H_2O_2. This in situ forming hydrogel might be a desirable candidate for biomedical applications.

Keywords: Biomedical Applications; Gum Tragacanth; Horseradish Peroxidase; *In situ* Forming Hydrogel; Tyramine

1. Background

In situ forming hydrogels, compared with preformed hydrogels, can fill defects with all shapes, allow homogeneous incorporation of therapeutic molecules/cells and do not require surgical procedures for implantation; consequently, research efforts on biomedical applications of *in situ* forming hydrogels has recently increased (1-5). *In situ* forming hydrogels have been prepared by several physical or chemical cross-linking methods. Mechanical strength and stability of physically cross-linked hydrogels against physiological pressures are generally poor. On the other hand, chemically cross-linked hydrogels usually exhibit enhanced mechanical strength and better stability but will suffer from potentially harmful side reactions (4). Among these strategies, enzyme catalyzed chemical cross-linking is most desirable, because it can be performed at mild physiological conditions, without any unwanted side reactions. Additionally, enzymatic gelation can occur at adjustable and fast gelation times; thus localized drug delivery with decreased burst release and suitable cell distribution together with proper integration of the gel with the surrounding tissues can be ensured (4).

Until now, several natural polymers such as chitosan (6, 7), carboxymethyl cellulose (8, 9), dextran (10), hyaluronic acid (11-16), alginate (17) and gelatin (18-22) functionalized with tyramine or aminophenol have been used for the preparation of enzyme catalyzed cross-linkable hydrogels.

Implication for health policy/practice/research/medical education:
An enzyme catalyzed *in situ* forming hydrogel, based on the water soluble part of the gum tragacanth conjugated with tyramine was prepared and evaluated. This hydrogel is potentially a desirable injectable hydrogel for biomedical applications.

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The excellent biocompatibility, biodegradability, bioactivity and biological properties of the hydrogels fabricated using natural polymers, especially polysaccharides, are very advantageous for biomedical applications. However, each hydrogel system, prepared using a special natural polymer, has distinctive intrinsic properties including cell/tissue adherence, mechanical strength, swelling behavior and degradation rate, which may be appropriate for particular applications. Additionally, these properties can be adjusted by modification of common polymers or using new synthetic or natural polymers (19, 23-30).

Gum tragacanth (GT) is a heterogeneous highly branched anionic polysaccharide obtained from Asiatic species of Astragalus (31, 32). This gum consists of two major fractions; tragacanthic acid (tragacanthin, TGA) (water-soluble) and bassorin (water-swellable). It was suggested that, tragacanthin composition is based on linear chains of partially methyl esterified 1,4-linked α-D-galacturonic residues that has many side chains, composed of d-xylose, l-fucose, d-galactose and trace amounts of D-glucuronic acid, bonded to acidic backbone (33-36). Gum tragacanth has been generally recognized as safe at the 0.2–1.3% level in food stuffs in USA since 1961 (37). This gum is widely used in food, pharmaceutical, ceramic and paint industry. In the pharmaceutical industry, it is used as a suspending agent in oil-in-water emulsions, jellies and toothpastes, as a stabilizer in dermatological creams and lotions as well as a binding agent in the production of drug tablets (31). Since the past decade, this gum has been investigated as a retarding agent for sustained release of drugs (38) and membranes with possible application in drug delivery systems (39, 40). Recently, Fattahi et al.(41) showed that physically cross-linked ferric-tragacanth gels exhibit better cell adhesion properties compared to physically cross-linked ferric-alginate gels. This was attributed to the interactions of cells with some monosaccharaides on the side chains of tragacanth, especially L-fucose (41, 42). These desirable properties make tragacanth a potential candidate for biomedical applications. Despite advantages in the application of this gum, its biomedical applications have not been fully explored.

In this study, an enzyme catalyzed *in situ* forming hydrogel using tragacanth natural gum from *Astragalus fluccosus* was prepared and evaluated for possible biomedical applications. For this purpose, after tyramine-tragacanthin (TA-TGA) conjugation, *in situ* forming hydrogel was prepared via an enzyme catalyzed cross-linking reaction. Next, the gelation time, swelling/degradation behavior and mechanical properties of the hydrogel and viability of the cells encapsulated within these hydrogels were investigated.

2. Objectives

The main objective of the present study was to evaluate primarily the possibility of using tragacanth natural gum based hydrogels for biomedical applications. To our knowledge, this is the first report on the preparation and characterization of *in situ* forming hydrogel using gum tragacanth.

3. Material and Method

3.1. Materials

Iranian gum tragacanth was collected from *Astragalus fluccosus* plants, growing in Shahrood, located north of Iran. The raw gum was ground and sieved to obtain powder with a mesh size between 160 and 300 μm. The gum was purified by dialyzing against deionized water for 48 hours. The purified product was freeze-dried and kept in well-sealed plastic bags for future use. One gram of tragacanth powder was added to 100 ml of deionized water. The mixture was stirred intensively for 3 hours and then gently over night at room temperature to ensure complete hydration. The mixture was then centrifuged for 90 minutes at 5000 rpm to separate soluble and insoluble fractions. Both fractions were dialyzed against deionized water for 48 hours, then freeze-dried, sealed in ziped plastic bags and kept at room temperature for future use.

Acridine orange (AO), propidium iodide (PI), (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), tyramine hydrochloride (TA.HCl), horseradish peroxidase (HRP, Type VI-A, 180 $U.mg^{-1}$) and 4-morpholinoethanesulfonic acid (MES) were purchased from Sigma-Aldrich company (St. Louis, MO, USA) and used without further purification. All other chemicals, solvents and reagents were of analytical grade.

3.2. Synthesis and Characterization of Tyramine-Tragacanthic Acid (TA-TGA) Conjugate

TGA was dissolved in 0.1 M MES buffer at 1% (w/v) solution concentration. pH of the solution was adjusted to 6.1±0.1 by addition of 2M NaOH. After addition of Tyramine, HCl (0.40 $g.g^{-1}$ TGA) solution was gently stirred for 30 minutes. Then, EDC (0.60 $g.g^{-1}$ TGA) and NHS (0.30 $g.g^{-1}$ TGA) were added and the solution was stirred gently at room temperature. At specific reaction times (6, 9, 12 or 24h), the reaction mixture was dialyzed versus 0.1 M NaCl for 24 hours and then deionized water for 24 hours. During the dialysis period, dialyzing medium was replaced every 12 hours. Finally, the product was freeze-dried and kept in well-sealed plastic bags for future use. The degree of tyramine substitution was measured using ultraviolet-visible spectrophotometry (UV/Vis Varian Cary 100, USA), according to the method described by Sakai et al. (21). Briefly, the absorbance of functionalized tragacanth solution in phosphate buffered saline (PBS) at 0.1% (w/v) concentration was measured at 275 nm. Next, the degree of tyramine substitution was estimated using the calibration curve obtained by measuring the absorbance of known concentrations of tyramine in PBS.

3.3. Hydrogel Preparation and Gelation Time

Firstly, different amounts of TA-TGA were dissolved in PBS overnight to ensure complete hydration. Fresh H_2O_2 and HRP solutions in PBS were prepared, immediately prior to hydrogel preparation. Next, appropriate volumes of the polymer, HRP and H_2O_2 solutions were mixed using a double syringe (1:4 volume ratios) equipped with a mixing chamber (MEDMIX, Switzerland). Gelation time was determined using the vial tilting method. No flow within 1 minute upon inverting the vial was regarded as the gel state.

3.4. Swelling and Degradation

In vitro swelling study was performed immediately after hydrogel preparation. The hydrogel (0.5 ml) was weighted (Wi) and 2 mL of PBS was placed on top of the gel. The media was incubated at 37 °C and shaken at 100 rpm. At specific time intervals, the buffer solution was removed and the hydrogel was weighted. The remaining gel at each time intervals was calculated using the following expression:

Remaining gel at time t: $R(t)=W_t/W_i$

where W_t and W_i are the sample weight at time t and immediately after preparation, respectively. Each experiment was repeated three times.

3.5. Rheological Analysis

All rheological measurements of the hydrogels were performed with an MCR 301 rheometer (Anton Paar GmbH, Graz, Austria) using parallel plate (25 mm diameter) configuration at 37 °C in the oscillatory mode. TA-TGA/HRP solution was immediately mixed with H_2O_2 solution using a double syringe equipped with a mixing chamber. After injection, the upper plate was immediately lowered to a measuring gap size of 1 mm, and the measurement was initiated after 2 minutes. To prevent evaporation, a layer of oil was introduced around the polymer sample. A frequency sweep on the hydrogels was performed from 0.1 to 100 Hz at 0.1% strain. Each experiment was repeated three times.

3.6. Cell Viability

The *in vitro* toxicity of GT and TA-TGA was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay. Firstly, human mesenchymal stem cells (hMSCs) or Caco-2 cells were seeded on a 96 well plate with a density of 10000 cells per cm^2. After 24 hours, 200 μl sterilized tragacanth solution in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) was added and cells were incubated at 37 °C. After 24 and 72 hours, the polymer solution was replaced by filter sterilized MTT solution in PBS buffer. After 3 hours of incubation at 37 °C, the MTT solution was removed. Then, 200 μl of dimethylsulfoxide (DMSO) was added to each well and pipetted three times. Optical densities of solutions were measured spectrophotometrically at 570 nm using an ELISA reader. Fifty percent cytotoxic concentration (CC50) was defined as the polysaccharide concentration that reduced the number of viable cells by 50% compared with the control without polysaccharide addition. Each experiment was repeated three times.

Viability study on the hMSCs encapsulated in the hydrogels was performed using the live/dead assay. To prepare cell-encapsulated hydrogels, immediately before hydrogel preparation, hMSCs were suspended in the initial sterile polymer solution (2×10^5 Cells.ml^{-1} hydrogel). Hydrogels were then prepared using the same procedure as in the absence of cells. All of the steps were carried out in sterile conditions. The cell-encapsulated hydrogels were cultured under standard culture conditions. After 2 and 288 hours of incubation, hydrogels were washed twice using sterile PBS and stained with acridine orange/propidium iodide. After 5 minutes, samples were rinsed twice with PBS and numbers of live (stained green) and dead (stained red) cells were counted by fluorescent microscopy. The average of ten measurements was taken as the cell viability percentage.

3.7. Statistical Analysis

Experimental results are presented as mean ± standard deviation. The difference between groups were tested by the analysis of variance (ANOVA). Differences were considered statistically significant at $P \leq 0.05$.

4. Results

4.1. Synthesis and Characterization of Tyramine-Tragacanthic Acid (TA-TGA) Conjugate

The proton nuclear magnetic response (H NMR) spectra of TGA and TA-TGA are shown in Figure 1. The degree of TA substitution on polysaccharide units, estimated by UV-Vis spectrophotometry, varied between 1.8 and 3.0% (w/w) depending on the reaction conditions. The degree of substitution did not significantly increase after 9 hours from the conjugation reaction ($P \leq 0.05$).

4.2. Hydrogel Formation and Gelation Time

A schematic representation of the cross-linking reaction of TA-TGA in the presence of HRP and H_2O_2 and pictures taken with a digital camera from the prepared hydrogel are shown in Figure 2.

The gelation time of TA-TGA as a function of polymer and HRP concentrations is shown in Figure 3. The gelation time decreased with increasing polymer and HRP concentrations. These phenomena can be attributed to the increased tyramine group concentration by increasing polymer concentration and to the increased rate of H_2O_2 decomposition and production of phenoxy radicals by increasing HRP concentration, respectively.

4.3. Swelling and Degradation Behavior

The *in vitro* swelling/degradation behavior of the TA-TGA hydrogels in PBS buffer (pH~7.4) is shown in Figure 4. The weight of all hydrogels increased initially due to the adsorption of water molecules by the hydrogel network for the complete hydration of polymer chains, and then increased slightly for several days due to degradation of some cross-linkage points, which lead to expansion of the hydrogel network. Later, the weight of the hydrogels decreased due to extra degradation of the hydrogel network with consequent polymer dissolution.

As shown in Figure 4, the rate of swelling and subsequent disintegration decreased by increasing HRP and H_2O_2 concentrations. This is due to the increased cross-linking density, which increases resistance against swelling and disintegration.

Hydrogels, which were prepared at higher polymer concentrations disintegrated at slower rates compared to hydrogels prepared at lower polymer concentrations due to the formation of denser hydrogel networks, which resist against disintegration. For example, at HRP concentration of 5.0 U/ml and H_2O_2 concentration of 15.0 mM, the hydrogels prepared with 1.0% (w/v) TA-TGA degraded in less than 35 days, but hydrogels prepared at 2.5% (w/v) TA-TGA remained stable for more than 60 days.

4.4. Rheological Analysis

The storage modulus of the hydrogels increased with increasing polymer concentrations ($P \leq 0.05$) due to increased cross-linkage density at higher polymer concentrations. For example, by increasing polymer concentration from 1.0 to 2.5% (w/v), storage modulus of the corresponding hydrogel increased from 253±12 to 615±22 Pa. Damping factors of the hydrogels were in the range of 0.003-0.03, which decreased by increasing polymer solution concentration. According to the frequency sweep analysis, hydrogels exhibit constant G' and G", independent of frequency ranging from 0.1 to 80 Hz. According to Lee et al. (11) the storage modulus of tyramine-functionalized hyaluronic acid is in the range of 10 to 4000 Pa.

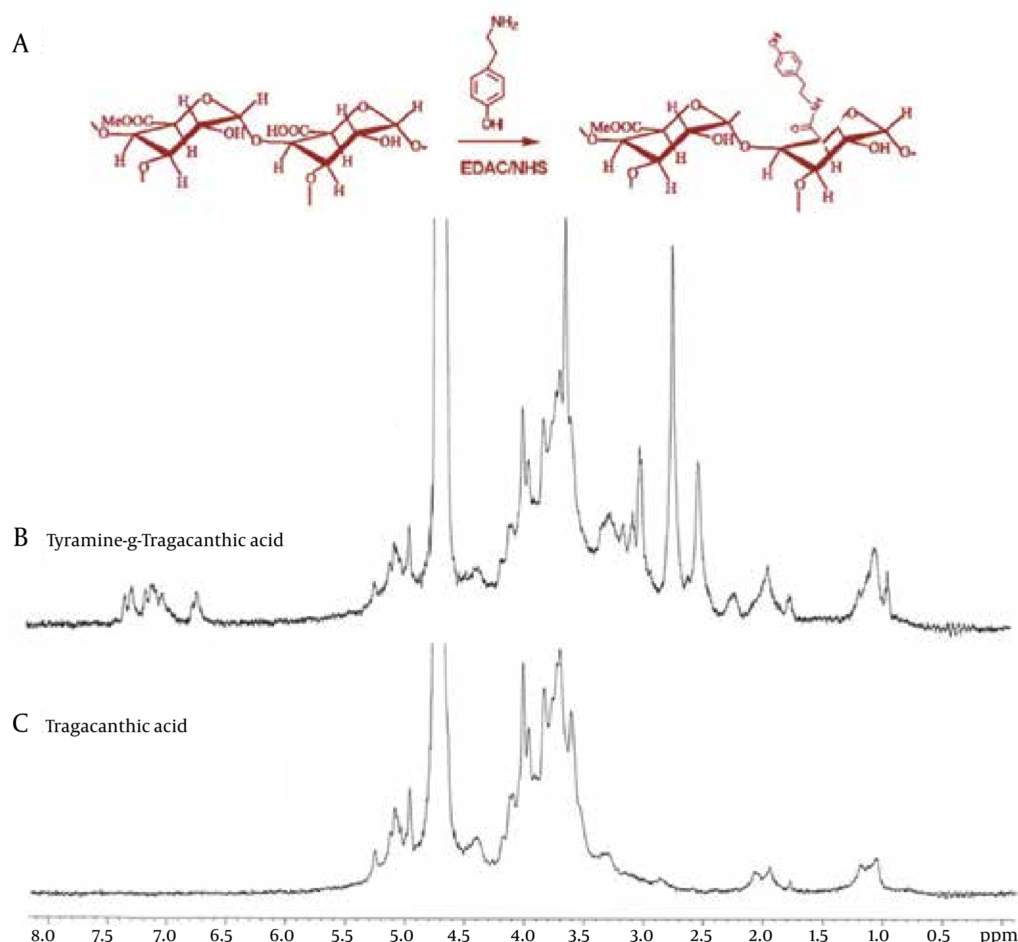
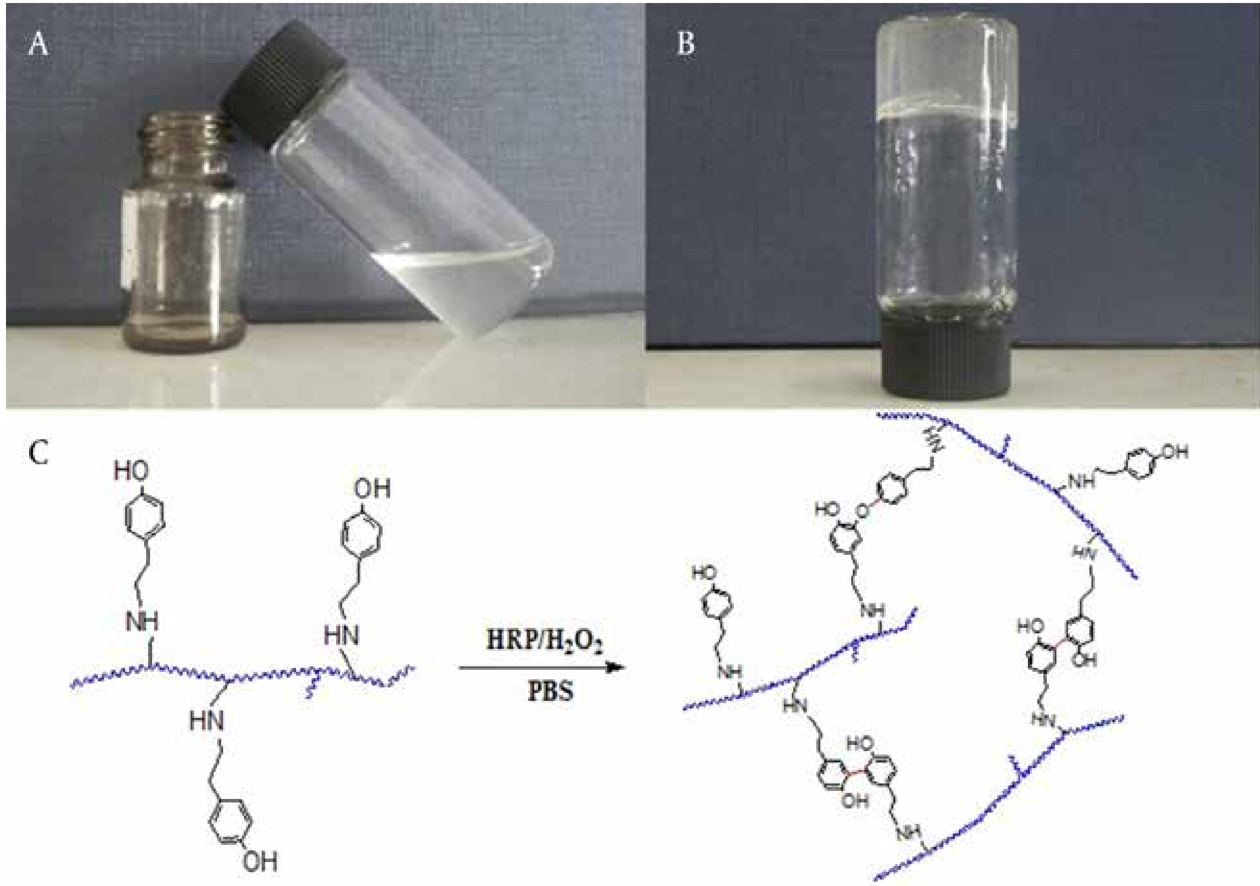


Figure 1. A) Reaction Scheme for the Synthesis of TA-TGA Conjugate, and the H NMR spectra of: B) TA-TGA and C) TGA

Figure 2. Schematic Representation of Enzymatic Gelation of TA-TGA and Pictures Taken from a Prepared Hydrogel



A) TA-TGA solution, B) TA-TGA hydrogel, C) Schematic representation of enzymatic gelation of TA-TGA in the presence of HRP and H_2O_2.

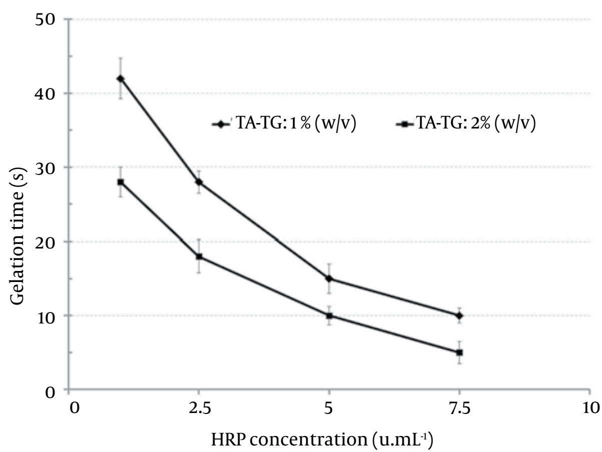


Figure 3. Effect of Polymer and HRP Concentrations on the Gelation Time of TA-TGA.

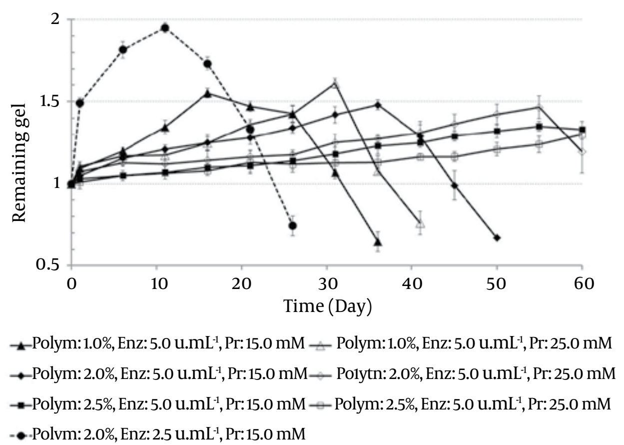


Figure 4. Swelling/Degradation Behavior of the TA-TGA Hydrogels.

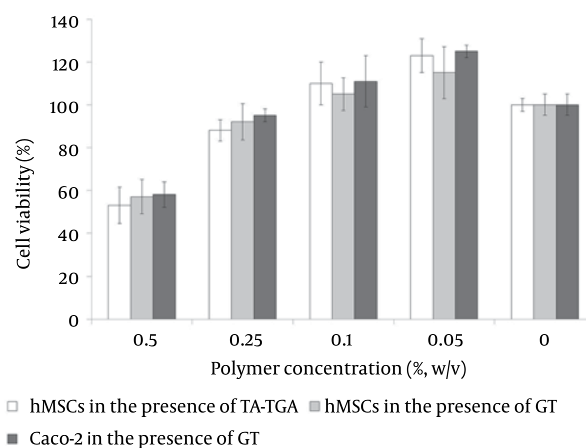


Figure 5. Effect of GT and TA-TGA Concentrations on the Viability of hMSCs.

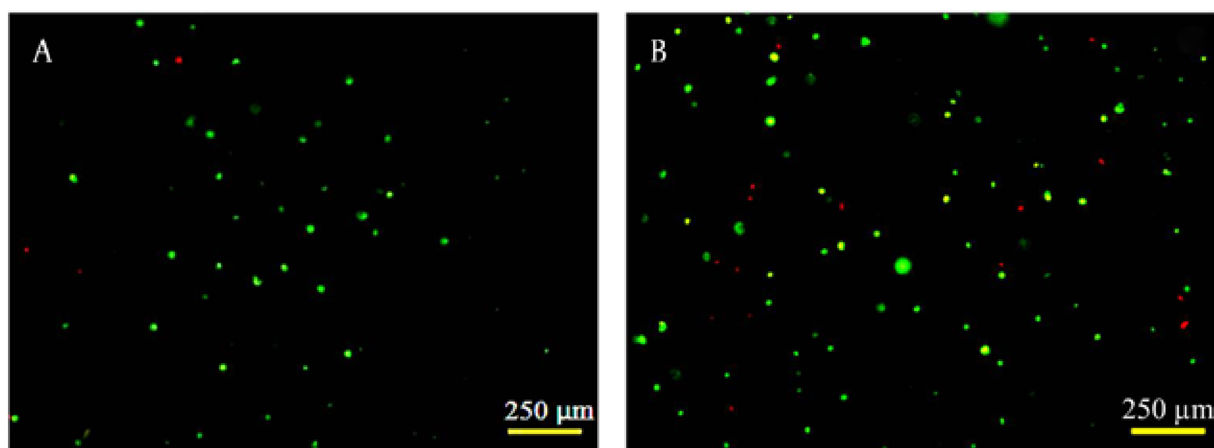


Figure 6. Fluorescence micrographs of encapsulated hMSCs, in the TA-TGA hydrogels prepared with 15.0 mM H_2O_2 after A) 2 hours and B) 12 days of incubation. Live cells were stained as green and dead cells as red.

4.5. Cell Viability

The effect of GT and TA-TGA concentrations on the viability of cells after 72 hours of incubation is shown in Figure 5. GT showed a dose-dependent effect on the viability of both hMSCs and Caco-2 cells. After 72 hours of incubation, the viability of hMSCs and Caco-2 cells slightly improved at GT concentrations of less than 0.1% (w/v). In addition, at concentrations $\leq 0.1\%$ (w/v), TA-TGA showed positive effect on the viability of hMSCs. According to these results, the CC50 of GT (for both hMSCs and Caco-2 cells) and TA-TGA (for hMSCs) were greater than 0.5% (w/v).

Lucyszyn et al. (43) reported that the CC50 of xyloglucan is greater than 0.33% (w/v). According to Kean & Thanou (44), the CC50 of chitosan is in the range of 0.021 to 0.25 mg/ml depending on the molecular weight and degree of acetylation.

The percentage of viable cells after 2 and 288 hours of incubation of hydrogels at 37 °C in the presence of 5% CO_2 was measured using a live/dead assay where cells were stained with two fluorescent dyes to find the ratio of live to dead cells within the hydrogels. As expected, the viability of encapsulated cells decreased with increasing H_2O_2 concentration (data not shown). According to Park et al. (27) almost all osteoblast cells seeded in tetronic-succinic anhydride-tyramine hydrogel fabricated at H_2O_2 concentrations lower than 18.5 mM were viable after 2 hours of incubation. As shown in the fluorescent microscopy image of stained hydrogels (Figure 6), approximately 93% of the encapsulated hMSCs in the hydrogels, prepared with 2.0% (w/v) TA-TGA, 15.0 mM H_2O_2 and 5.0 u/ml HRP, were still viable after 2 hours of incubation. The viability of these cells decreased to 84.0±3.0% after 12 days of incubation.

5. Discussion

As mentioned previously, the backbone of the soluble part of gum tragacanth, named tragacanthic acid, con-

sists of partially esterified α-D-galacturonic acid repeated units (35). Gum tragacanth exuded by *A. fluccosus*, used in the present study, had the highest soluble: insoluble ratio of 3.16 and relatively low methyl esterifying degree (29-34 mg/g of gum), compared to other Iranian tragacanth gums (16, 25). Therefore, in the present study, tyramine functionalized tragacanthic acid was prepared by formation of amide bonds between the carboxyl groups on tragacanthic acid and amine groups on TA using conventional carbodiimide/active ester mediated coupling reaction in buffered aqueous media. Comparing the H NMR spectra of TGA and TA-TGA (Figure 1), presence of new peaks at δ 6.7-7.3 and δ 2.7-3.1 in the H NMR spectrum of TA-TGA, relative to the presence of TA groups, indicates that TA-TGA conjugate was successfully synthesized. Likewise, comparison of the UV-Vis absorbance peaks of TA-TGA and TGA solutions showed that a rise of absorbance at 275 nm can be attributed to the tyramine conjugation. TA-TGA hydrogel is formed through covalent cross-linkage between tyramine functionalized tragacanthic acid molecules due to the generation of phenolic oxygen radicals in the presence of HRP and H_2O_2 (10). The fast gelation of the TA-TGA hydrogels is desirablefor injectable drug delivery/tissue engineering devices, since slow gelation *in vivo* may result in failure of gel formation or diffusion of hydrogel precursors and laden bioactive molecules to surrounding areas as well as settling out of the cells before gelation (8, 10, 19, 29). The gelation time of this hydrogel is comparable with those reported for other tyramine functionalized biopolymers, such as tyramine-dextran (10) and tyramine-gelatin (21).

The rate of hydrogel degradation decreased by increasing HRP, H_2O_2 and TA-TGA concentrations, due to the increased cross-linking density, which increases resistance against swelling and disintegration. Rheological analysis results confirmed the elastic characteristics of the hydrogels. According to these results, the *in situ* forming TA-TG hydrogel is a soft hydrogel with elastic characteristics suitable for some biomedical applications such as drug delivery and soft tissue engineering. The gelation time, swelling/degradation behavior and rheological properties of the hydrogel could be adjusted by changing the polymer, HRP and H_2O_2 concentrations.

The obtained CC 50 values indicate that both TGA and TA-TGA have good cytocompatibility and can potentially be used as biocompatible polymers for biomedical applications. Hydrogen peroxide has serious cytotoxicity effects on mammalian cells, depending on its concentration, thus to maximize the viability of encapsulated cells, H_2O_2 concentration must be minimized. The results of cell viability are promising for application of TA-TGA as a biocompatible cell carrier in tissue engineering. According to our results, the invented tragacanth based *in situ forming* hydrogel might be suitable for biomedical applications.

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