Synthesis, characterization and toxicity assessment of newly polymeric nanoparticle

L-glutamic acid-g- p(HEMA)

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Abstract

Preparation of poly(HEMA) based polymeric nanoparticles allows to create new biomedical applications as drug delivery systems and hence it leads to toxicological studies. The aim of the study was to characterize and to evaluate the toxicity for its biocompatibility of newly synthesized L-glutamic acid-g-p(HEMA) polymeric nanoparticle. It was synthesized with surfactant free emulsion polymerization and grafting techniques. Grafting yield was calculated 58 %. Morphology was verified as nearly spherical in shape by scanning electron microscopy (SEM). Atomic force microscope (AFM) images were showed a roughness area in surface topography of nanoparticle. Zeta analysis showed that particle have an average size of around 194.6 nm and the zeta potential is a negative value which is -18 mV. The sample was scanned from 750 to 4000 cm^{-1} for Fourier transformed infrared (FT-IR) spectroscopy and the characteristic peaks of stretching band were observed in spectrums. The polymeric nanoparticle showed 46% swelling ratio. p(HEMA) and L-glutamic acid-g-p(HEMA) have not been showed the cytotoxic effect on human bronchial epithelial cell line (16HBE) and human monocytes cell line (THP-1) of 24 h exposure with WST-1 and LDH assay. It has not genotoxic potential in comet assay and also it was not caused mutagenic effects on *Salmonella typhimurium* TA 98, TA 100, TA 1535 and TA 1537 strains in Ames test. L-glutamic acid-g-p(HEMA) has 2% hemolytic activity on erythrocytes at 160 $\mu\text{g/ml}$. In cell migration test, the percentage closure difference was calculated as 21%. There is not irritation effect with HET-CAM test. The polymeric nanoparticle was found to be biocompatible with these analyzes and it may a potential to use as drug delivery systems.

Key words: L-glutamic acid-g-p(HEMA), polymeric nanoparticle, cytotoxicity, genotoxicity, hemolysis

1. Introduction

Polymeric nanoparticles (PN) are widely used in many applications as tissue engineering, drug and gene delivery systems [1,2]. Various biocompatible polymeric nanoparticles are conjugated with drugs in accordance with their chemical structure and are used as a delivery system in several diseases such as cancer [3], diabetes [4], tuberculosis [5]. The size distribution and surface chemistry of polymeric structures vary according to the synthesis method and the conjugates offered during grafting. Polymer grafting procedure is a technique to modify the polymer's physical properties by chemical binding of e.g. proteins, peptides, amino acids which have been utilized as controlled-release carriers for drugs adsorbed onto their surfaces [6]. The size distributions, surface charge, surface modifications of nanoparticles are important to determine their interaction with the cells. While synthesizing new polymeric material, toxicological response of the material should be investigating to determine a safe dose [7], since small changes in physio-chemical parameters as particle shape, size, surface charge can affect toxic responses of polymeric nanomaterials [8].

One of commonly used polymeric nanomaterial is poly (2-hydroxyethyl methacrylate)-p(HEMA), with applications in drug delivery, hydrogels in artificial cornea, contact lenses and bone composite materials [9-11]. The presence of hydroxyl, carboxyl groups and methyl groups makes poly (HEMA) a multipurpose vehicle [12]. The amino acid L-glutamic acid has an amine and two carboxyl moieties. The presence of these groups in the structure of L-glutamic acid allows the binding and surface modifications of different functional groups. The possible toxic effects of each new synthesized material should be investigate very well considering the next applications such as drug delivery. It is not correct to make generalizations about the toxicities of nanoparticles. It should be considered that each new material will behave differently. So, the aim of this study is synthesis, characterization and toxicity/biocompatibility evaluations of newly synthesized material L-glutamic acid-g-p(HEMA) polymeric nanoparticle.

2. Material and Methods

2.1. Synthesis of L-glutamic acid-g-poly(HEMA) polymeric nanoparticle

Surfactant free emulsion polymerization was carried out with minor modifications according to Turkcan et al (2013) [13]. Polyvinyl alcohol (PVA) (0.5 g) as a stabilizer was dissolved in 25 mL deionized water. Then, the hydroxyethyl methacrylate (HEMA)/ Ethylene glycol dimethacrylate (EGDMA) mixture 0.6 mL/0.3 mL was added to the dispersion. Potassium peroxydisulfate (KPS) 0.0198 g was dissolved in 45 mL distilled water. Finally, KPS (as initiator) was added to the monomer phase and polymer emulsion medium was treated with nitrogen gas about 1–2 min for removing dissolved oxygen. Polymerization was carried out in a shaking bath at 70 °C for 5 h. After the polymerization, poly(HEMA) nanoparticles were washing with ethanol and water two times to remove the unreacted monomers. After last washing step the nanoparticles were precipitated by centrifugation (Eppendorf, Minispinplus, Germany) at 14.500 rpm for 30 minutes.

In order to prepare L-glutamic acid grafted poly(HEMA) nanoparticle (Fig 1), 20 g of dried poly(HEMA) (fraction W1) was transferred into the reactor. L-glutamic acid solution mixture was prepared with 3 g L-glutamic acid (fraction W2) in 50 ml of tetrahydrofuran and 1.4 g of NaH as a catalyst were added in the mixture and constantly gentle (magnetic) stirring at 40°C for 24 h. Thereafter, nanoparticles were washed with water and methanol respectively, in order to remove unreacted molecules and dried in vacuum for 24 h (fraction W3). They were stored at 4°C until use [14]. The grafting yield percentage was determined by calculating the weight using following equation [15];

$$\text{Grafting yield (\%)} = \frac{W3 - W1}{W2 - W1} \times 100$$

W1: initial weight of the sample, W2: weight of the grafted sample, W3: weight of the grafted sample after washing

2.2. Characterization of L-glutamic acid-g-poly(HEMA) polymeric nanoparticle

2.2.1. SEM (Scanning electron microscopy) imaging

Morphology and size distribution of the polymer was examined by scanning electron microscopy (SEM Philips XL 30S FEG, IZTECH, Izmir-TURKEY).

2.2.2. ZETA size and potential analyses

The average L-glutamic acid-g-poly(HEMA) polymeric nanoparticle size and distribution were determined by Zeta Sizer (Malvern ZETA ZS, England). The measurements of the zeta potential were carried out at various pH values (pH 4–10) to observe the effect of pH on the zeta-potential of the nanoparticle.

2.2.3. FT-IR (Fourier transformed infrared) spectroscopy

Fourier transformed infrared (FT-IR) spectroscopy of the particle was obtained using a FT-IR spectrophotometer (IRTracer-100, Turkey). The sample was scanned from 750 to 4000 cm^{-1} and recorded with characteristic peaks of poly(HEMA) and L-glutamic acid-g-p(HEMA).

2.2.4. AFM (Atomic force microscopy) characterization

Atomic force microscopy (AFM) analyses was carried out under ambient conditions (24 °C, 40% RH) in tapping mode. The scanning speed of the tip was 1 Hz and the scanning area was 10 μm ×10 μm (BRUKER Dimension Edge with ScanAsyst).

2.3. Swelling test

The water absorbing capacity of polymeric nanoparticle was determined as described earlier with minor modifications [16]. The swelling of polymeric nanoparticle was evaluated by measurement of weight. The dry nanoparticles were immersed in distilled water and phosphate

buffered saline (PBS) at pH (7.4) for 24 h at room temperature until an equilibrium was reached. Samples were taken at predetermined time intervals by filtration, blotted with filter paper for the removal of the surface water, and weighed. The swelling ratio was calculated using the following equation;

$$\text{Swelling ratio (\%)} = \frac{Ws - Wd}{Wd} \times 100$$

where, Ws is weights of swollen sample, Wd is weights of dry sample.

2.4. *In vitro* Biocompatibility Studies

2.4.1. Cell Culture and Cytotoxicity Assay

The human bronchial epithelial cell line (16HBE) and the human monocytic cell line (THP-1) were kindly provided by Dr. Gruenert (University of California, San Francisco, USA). THP-1 cell line was cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin/ streptomycin, 2 mM L-glutamine and 2.5 µg/ml fungizone and 16HBE cell line was cultured in Dulbecco's modified eagle medium DMEM/F12 supplemented in 5% of FBS, 100 U/ml penicillin/ streptomycin, 2 mM L-glutamine and 2.5 µg/ml fungizone. The cells were incubated at 37°C in a 100% humidified air containing 5% CO₂. The culture was changed every 2 or 3 days until cells were confluent.

Cytotoxicity tests were performed using standard protocol [17]. The cytotoxicity was determined by in vitro water soluble tetrazolium salt (WST-1) assay which measures the activity of mitochondrial reductase and lactate dehydrogenase (LDH) assay which measures the leakage of LDH from the cytosol to the culture membrane. The cultured cells were treated with different concentrations (6.25 µg -1 mg/ml) and incubated for 24h at 37 °C. In WST-1 assay, the optical density was measured at 450 nm in a multi-well plate reader (BIORAD, Model680XR microplate reader). The relative viability was calculated against the negative

controls (untreated cells) and demonstrated in percentage. The % viability was determined as formulated below [18]:

$$\%Viable\ cells = \frac{[(the\ absorbance\ of\ the\ treated\ cells) - (the\ absorbance\ of\ the\ blank)]}{[(the\ absorbance\ of\ the\ control) - (the\ absorbance\ of\ the\ blank)]} \times 100$$

In LDH assay, the change in absorbance (depletion of NADH) was measured by spectrophotometer at 340 nm for 3 min with 15 s interval. Finally, the cell viability was calculated according to the formulae:

$$\% Viability = \frac{slope\ of\ lysate}{(slope\ of\ lysate + slope\ of\ supernatant)} \times 100$$

$$Relative\ viability = \frac{(sample\ viability)}{(untreated\ control\ viability)} \times 100$$

The cells which treated with different concentrations were examined by inverted light microscopic to observe potential morphological changes.

2.4.2. In vitro cell migration assay

The cell migration test is used for wound healing study, also used to observe the morphological characteristics of the cell during migration according to the applied substance. The test was carried out with IBIDI culture-insert 2 well kit (Cat No: 81176) which provides two cell culture reservoirs when placed on a cell culture dish surface. The reservoirs are separated by a 500 μ m thick wall. According to the test procedure, 5×10^4 / ml cells were planted with 16HBE culture and their media were changed with serum free medium after 24 hours of incubation. Cells were observed and photographed every hour from 0. time point when exposed to polymeric material (0.1 mg/ml) under the microscope. Seeding cells in both reservoirs and removing the silicon insert from the surface results in two defined cell patches separated by a cell free gap of 500 μ m width. Field measurements were performed with ImageJ program by tracing the wound perimeter and calculating percentage closure.

2.4.3. Bacterial Reverse Mutation Assay (Ames Test)

Bacterial reverse mutation assay was performed in *S.typhimurium* TA98, TA100, TA 1535 and TA 1537 strains according to the OECD Guideline 471 [19]. Experiments were performed, using triplicate plates, in the presence and absence of metabolic activation by Aroclor 1254-induced (500 mg/kg body weight) Swiss albino mice liver post-mitochondrial fraction (S9). The post mitochondrial fraction was used at a concentration of 10 % v/v in the S9 mixture. Negative and positive controls were tested in all strains in both experiments. Benzo[a]pyrene (5 µg/plate) and sodium aside (10 µg/plate) were used as positive controls. Fresh cultures of tester strains were grown to approximately 10^9 cell/mL in 5 mL nutrient broth (Oxoid Ltd, Basingstoke, Hampshire, UK). The cultures were incubated for 10–12 h at 37 °C in an incubator in order to insure adequate aeration. The recommended maximum test concentration, 5 mg/plate, was applied to all tested strains according to the OECD Guideline 471 [19]. The mixture containing chemicals and bacteria with or without S9 was vortexed and pre-incubated at 37 °C for 30 min. It was then plated in 2 mL of top agar on glucose supplemented minimal agar. After 48 h of incubation at 37 °C, revertant colonies were counted. The results of the test were presented as mean $\pm$ standard deviations (SD). Comparisons were made between control and treatment groups using one-way analysis of variance (ANOVA) followed by Dunnett's test. Values of $p < 0.05$ were regarded as statistically significant. Statistical analyses were performed using SPSS for Windows V.11.0.

2.4.4. Comet Assay

DNA strand breaks were analyzed on 16HBE and THP-1 cell lines with Trevigen CometAssay kit (4250-050-K) according to the provided protocol. The cells were exposed to 6,25-25µg/ml L-glutamic acid-g-p(HEMA) polymeric nanoparticle for 24 h. Methyl Methanesulphonate (MMS) was used as positive control. After electrophoresis the DNA was stained with SYBR-gold dye. Slides were examined using Olympus fluorescence microscope at a $\times 10$

magnification. For each experimental condition 100 randomly chosen cells from two duplicate slides were examined.

While evaluating the comets, the interaction of the SYBR-gold dye with nanoparticle resulted in extra irradiation in the background on the slides, which gave misleading results in the assessment of DNA damage (% tail DNA) by Casplab-Software. Therefore, instead of software based analyses, DNA damage was expressed as valid method Arbitrary Units (AU) based on the classification of comets into five categories (0–4) as proposed in earlier studies [20-23]. In total 100 comets were scored visually according to the length of the tail and given a value of 0, 1, 2, 3 or 4 (0: undamaged, 1: little damaged, 2: medium damaged, 3: damaged, 4: maximally damaged). The AU were calculated as follows: $AU = (0 \times N_0) + (1 \times N_1) + (2 \times N_2) + (3 \times N_3) + (4 \times N_4)$, where N_i is the number of nuclei scored in each category [20,22]. Thus, the overall score ranges from 0 (all cells undamaged) to 400 (all cells maximum damaged) arbitrary unit. Slides were analyzed by one observer to minimize the scoring variability. One-way ANOVA was performed for the genotoxicity analysis, using GraphPad Prism7.

2.4.5. Hemolysis Assay

The protocol was approved by the Ege University, Local Ethical Committee of Animal Experiment (22.04.2015 date, 2015-044 number). In the study, we used the international ASTM standard practice F 756-00 [24] with minor modifications to determine of hemolytic properties. This assay to determine the percentage of nanoparticle induced hemolysis when red blood cells are exposed to test substance [25]. New Zealand rabbits (weighing 2000–2500 g) were obtained from Bornova Veterinary Control and Research Institute, Izmir, Turkey. The fresh blood was collected in anti-coagulated with Li-heparinized tubes from rabbits. The hemolysis was tested at four concentrations in the range of 20-160 µg/ml. Triton X-100 at a stock concentration of 1% (10 mg/mL) was used as positive control and PBS was used as the

negative control. The calibration standards and quality controls for both pre-study and study validation were prepared as described in the experimental procedure. The amount of hemoglobin released into the supernatant was quantified at absorbance of 540 nm. According to the criterion in the ASTM standard, percent hemolysis less than 2 means the test sample is not-hemolytic; 2-5 % hemolysis means the test sample is slightly hemolytic; and >5% hemolysis means the test sample is hemolytic. The percentage of hemolysis was determined as follows [26];

$$\text{Hemolysis \%} = \frac{A_{\text{sample}} - A_0}{A_{100} - A_0} \times 100$$

Where A_{sample} is the absorbance of the sample, A_{100} is the absorbance of completely lysed red blood cells and A_0 is the absorbance of no blood sample.

2.4.6. HET-CAM (Hen's egg test chorioallantoic membrane) test

The HET-CAM test is one of the alternative tests offered for in vitro study of ocular irritation by EURL-ECVAM (European Union Reference Laboratory for Alternatives to Animal Testing) [27]. The potential irritancy of compounds can be observed with adverse changes that occur in the chorioallantoic membrane of the egg after exposure to test sample. The test was carried out according to the ICCVAM-Recommended test method [28]. Fertilized hen's eggs were obtained from Abalıoglu Facility (Izmir). Three eggs for each concentration weighing between 50 and 60 g were selected in the experiment. These eggs were incubated in incubator at a temperature of $37 \pm 0.5^\circ\text{C}$, 70 % humidity for 7 days. On the 7th day, a window (2×2 cm) was made on the equator of the eggs through which formulations (0.3 mL) were instilled directly onto the CAM surface and left in contact for 0.5, 2 and 5 minutes. The membrane was examined for vascular damage, and the time taken for injury to occur was recorded. An 0.9% NaCl solution

was used as negative control, 0.1 N NaOH was used as positive control. The irritation severity (IS) for a period of up to 5 min was scored as;

$$IS = [(301-h) \times 5]/300 + [(301-l) \times 7]/300 + [(301-c) \times 9]/300$$

Where, h is the time of vascular hemorrhage; l is the time of first vascular lysis; and c is the time of first vascular coagulation. Irritation classification based on IS: 0.0–0.9, non-irritation; 1.0–4.9, slight irritation; 5.0–8.9, moderate irritation; and 9.0– 21.0, severe irritation.

3. Results

3.1. Synthesis of L-glutamic acid-g-p(HEMA)

L-glutamic acid-g-p(HEMA) was successfully synthesized by surfactant free emulsion polymerization and grafting methods. The grafting yield of L-glutamic acid-g-p(HEMA) was calculated as 58 %.

3.2. Characterization results

Surface morphology of L-glutamic acid-g-p(HEMA) is determined by SEM analysis (Fig 2). Morphological structures of the polymeric nanoparticle were nearly spherical in shape. The surface topographic image with AFM imaging of the L-glutamic acid-g-p(HEMA) polymeric nanoparticle is shown in Fig 3. L-glutamic acid-g-p (HEMA) had a general rough surface topography and Rq (RMS roughness) and Ra (roughness average) values were calculated as 68.8 nm, 49.4 nm, respectively. The copolymers have an average size of 194.6 nm and polydispersity value (PDI) was 0.638 (Fig 4). The polymeric nanoparticle showed differences in pH sensitive size in measurements taken at different pHs (pH 4-10) which is showed a narrow distribution in pH 4 and a large size distribution due to increasing pH value. (Fig 5). The zeta potential showed a negative surface charge of -18 mV. In the FT-IR spectroscopy, the sample was recorded from 750 to 4000 cm⁻¹ (Fig 6). In the spectrum, a few characteristic peaks were

observed: O-H stretching band at 3500-3400 cm^{-1} ; the n(C-O) groups at 1722 cm^{-1} ; the n(C-H) groups at 2986 cm^{-1} . The $-\text{NH}_2$ peak (1586-1611 cm^{-1}) is observed in the L-glutamic acid structure, and NH- group in the grafted polymer is seen at 1540 cm^{-1} . The $-\text{COOH}$ group which is specific also for glutamic acid (1720-1740 cm^{-1}) in the grafted polymer is clearly visible. As shown in Fig 7, the swelling ratio is plotted in water and phosphate buffered saline (PBS). While the swelling rate differed for suspensions in water and PBS, a maximum swelling of 46 % was noted after 15 minutes for both suspensions, hereafter no further swelling was observed.

3.3. Biocompatibility results

In the experiments to show the biocompatibility of L-glutamic acid-g-p(HEMA), we demonstrated that none of the tested concentration of L-glutamic acid-g-poly(HEMA) (up to 1mg/ml) induced cytotoxicity in both cell line used after 24hour incubation using WST-1 and LDH assays (Fig 8). The polymeric nanoparticles at 20,40, 80 and 160 $\mu\text{g/ml}$ concentration showed 0.4, 1.2, 1.7 and 2.0% hemolysis on erythrocytes, respectively. The results showed that the highest concentration used is slightly hemolytic (2%), (Fig 9). According to the cell migration test result; when the control group was compared with exposure group, the control was closed in 10 hours, the exposure was completely closed in 12 hours and the percentage closure was calculated as 21% which gives 2hour difference between control and exposure. (Fig 10).

L-glutamic acid-g-poly(HEMA) did not show mutagenic activity (even at 5000 $\mu\text{g/ml}$ concentration) in any of the bacterial strain tested under the condition with and without S9 mix. The test results are shown in Table 1. The comet assay results showed no statistically significant genotoxic effects on applied concentrations compare to negative control on 16HBE and THP-1 cell line (Fig 11). There is no irritation effect at applied concentrations of L-glutamic acid-g-p(HEMA) polymeric nanoparticle in HET-CAM test (Table 2, Fig 12).

4. Discussion

During the last decade, controlled polymerization techniques have led to a wide range graft copolymer structures with various functional groups [29]. In this study, we grafted poly-HEMA with L-glutamic acid because of the presence of two acid and one amine group, allowing the binding of different functional groups. L-glutamic acid-g-p(HEMA) was synthesized with surfactant free emulsion polymerization and grafting procedure described by Akgol et al. (2001) [13, 14], and it has an average particle size of 194.6 nm, a graft degree of 58%, and a zeta potential which is -18 mV. It has more carboxyl and hydroxyl groups which provides to negative surface to HEMA [30]. L-glutamic acid-g-p(HEMA) showed pH sensitivity as size distribution, which is a property that can be used for the development of drug delivery systems. Our synthesis yield is very well in line with Won Shin et al. (2015) who showed a graft yield of 53.1% and a particle size of 200 nm; these authors reported that the particle size decreased with an increasing grafting yield [8]. In addition, for increase the numbers of carboxyl groups in polymer structure, the molecule modifiers such as citric acid, glutamic acid or aspartic acid should contain two or more carboxyl groups [16]. In addition to this, it was known that the negative charge of the nanoparticle surfaces is due to the carboxyl groups of glutamic acid [31]. The AFM analysis gives an indication of the surface roughness; our samples showed very few valleys indicating the polymeric samples are homogeny in size. AFM allows non-destructive imaging of surfaces at the molecular scale [32]. It has been found that extremely low surface roughness could be attributed to a more homogeneous surface formation and more hydrophilic surface in the form of a hydrogel and also a higher water content provides less surface roughness [33,34,35].

The swelling state and swelling time of polymer contributes to its behavior as shape- and size-specific in mediums [36]. Also, the degree of swelling is known to vary depending on ambient conditions (temperature, pH) and the synthesis conditions of the particles. The

examined particles increased by 45% in volume within one quarter upon submerging in an aqueous solution. The rather low degree of swelling is desirable, since high swelling can cause cracking of the drug's external cap and unwanted drug loss [37]. However, it is known that there is a decrease in the swelling percentage of cross-linked polymers such as HEMA, because of the molecules of the cross linker are located between the monomer chains [38].

Even though each part of the newly synthesized nanomaterials is biocompatible, the toxicity of the composite molecule must be assessed because the different functional groups in the structure come together which can lead to a different effect. This is the first study, we synthesized and defined this polymeric nanoparticle and no previous comprehensive toxicity studies with this polymer were identified. The toxic effects at the cellular level can vary upon the cell types used [39]. In this study, the toxicity properties of the L-glutamic acid-g-p(HEMA) were carried out in several in vitro assays using different biological systems e.g. red blood cells, bacteria, adherent human 16HBE and non-adherent human THP-1 cells. We used serum-free cell culture medium during exposure since serum proteins influence the toxicological properties of nanoparticles [40]. In the assessment of cytotoxicity, no statistically significant effect was observed with both 16HBE and THP-1 cell line at different concentration, although some effects were noted with the 16HBE cell line at some of concentration in the LDH assay. Cytotoxicity is due to the solubility and absorption of the components, but also results from biological parameters such as cell type or culture and exposure conditions (eg cell density, particle concentration, ambient composition, temperature) used for research [41]. When we look at the literature for polymers cytotoxicity several observations are made, e.g.: polyethylenimine graft poly(ϵ -benzyloxycarbonyl-L-lysine) polymers exposed to CaCo-2 gut cells was evaluated showing low cytotoxicity at concentration at 1000 $\mu\text{g/ml}$ [42]; other polymers multi-armed poly(L-glutamic acid)-graft-OEI exposed to CHO and HEK293 cells showed low cytotoxicity depended on the molecular weight and surface charge; another study

showed that HEMA polymer prohibited sodium dodecyl sulfate toxicity in 3T3 fibroblasts cells [43]. Therefore, it is necessary to remark that it depends on the concentration and cell line to indicate biocompatibility.

The test of compatibility of nanomaterials with red blood cells which based membrane integrity is extremely important especially when nanoparticles are used intravenously [44]. Several studies showed that amines in nanoparticle, such as poly L-glutamic acid derivatives, can affect the toxicity [45,46,47]. On the contrary to these studies, we did not observe the hemolytic activity on red blood cells though it contains amine group.

The cell migration test allows to understand how long and how well a specific cell type can respond and migrate depending on the applied test substance [42]. Cell migration test is used in wound healing studies, but also can be used to observe the morphological characteristics of the cells during the migration after exposure test substance. In this study, when compared control and exposure groups, the closure difference was calculated as 2 hours and the closure percentage was calculated as 21% which means the exposure group was slower than control group. This may be because of nanoparticles features such as size and morphology, which has an effect on cell function and behavior, cell proliferation, migration as well as on cell uptake [48]. In addition, wound healing is a process involving fibroblasts and many anti-inflammatory cells at the tissue level. In most cases, healing is desirable, but an excess of scar tissue is an undesirable complication and may cause functional negative results at tissue and organ level. In this respect, the fact that the polymeric nanoparticle does not affect wound healing is seen as a rather beneficial feature.

Although the Ames test has been applied multiple times in nanomaterial testing, there is also criticism due to the limited uptake of nanoparticles through the cell wall [49] or attach to the external membrane and located inside the bacteria [50]. When examined the results of AMES test, the number of revertants is determined by counting the colonies seen in the plaques

and it is determined whether the substances are mutagenic or not according to the revertants colony number range given for each strain [51]. According to test result, polymeric nanoparticle didn't show mutagenic effect even at high concentration at all strains. It may be that the hydrophilic properties, physico-chemical characteristics of particles and also due to applied dose. The comet assay has been used for evaluation of the genotoxicity of some nanoparticles included in drug delivery systems with other particles as single-walled carbon nanotubes (SWCNT), titanium dioxide (TiO₂) and carbon black particles [52, 53]. The binding of the DNA dye with the particles resulted in the high background fluorescence and hindered automated scoring. For this reason, DNA damage (% tail DNA) was analyzed manually with consideration and classification of the comet tail with damage range [25,29]. According to comet assay results L-glutamic acid-g-p(HEMA) did not cause DNA-breaks. In the evaluation of genotoxicity of polymeric nanoparticle, AMES and comet assay results were supported each other with negative results.

The HET-CAM is a test which is use for determine irritation effect of samples. However, when given the intravenous route of administration of polymeric nanoparticles, the HET-CAM test has also been shown to be an important parameter for controlling the degree of vascular occlusion depending on the particle size [54]. In this study, especially this test was chosen by considering the use of HEMA in contact lenses. L-glutamic acid-g-p (HEMA) did not show hemorrhage, vascular lysis or coagulation effect, also it did not cause any irritation effect at applied concentrations. This results shows that L-glutamic acid-g-p (HEMA) has a potential for ocular delivery systems.

5. Conclusions

Characterization analyzes have supported that L-glutamic acid-g-p(HEMA) polymeric nanoparticles were synthesized successfully. The toxicological tests did not show significant negative result, suggesting that these L-glutamic acid-g-p(HEMA) polymeric nanoparticles are relatively safe and biocompatible.

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Declaration of interest

The authors declared no conflicts of interest.

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Table 1. AMES test results on *Salmonella typhimurium* strains

Concentration (μg / plate)	Mean <i>his</i> + revertants / plate ($\bar{X} \pm \text{SD}$)							
	TA 98		TA 100		TA 1535		TA 1537	
	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9
Distilled water	40 $\pm$ 9	35 $\pm$ 11	130 $\pm$ 43	116 $\pm$ 35	19 $\pm$ 4	12 $\pm$ 7	15 $\pm$ 8	18 $\pm$ 9
L-glutamic acid-g- p(HEMA) (5000 μg)	12 $\pm$ 5	23 $\pm$ 8	92 $\pm$ 6	36 $\pm$ 9	23 $\pm$ 10	25 $\pm$ 9	13 $\pm$ 8	17 $\pm$ 6
Benzo[a]pyrene (5 μg)	672 $\pm$ 45*	-	764 $\pm$ 57*	-	358 $\pm$ 62*	446 $\pm$ 58*	-	-
Sodium azide (10 μg)	-	935 $\pm$ 64*	-	1157 $\pm$ 72*	-	-	542 $\pm$ 68*	485 $\pm$ 58*

* Statistically significant from the control ($p < 0.05$).

Table 2. HET-CAM test scoring results

Sample	Concentration	Egg number	Effect	Score
L-glutamic acid-g p(HEMA)	5 mg/ml	3	No	0
	2.5 mg/ml	3	No	0
	1 mg/ml	3	No	0
Negative control	%0.9 (w/v) saline	3	No	0
Positive control	0.1N NaOH	3	Strong	>1

Figure Legends

Fig 1. Schematic illustration of L-glutamic acid-g-poly(HEMA)-red and black shows to structure of HEMA, green shows the structure of L-glutamic acid.

Fig 2. Scanning electron microscopy (SEM) images of L-glutamic acid-g-p(HEMA) (x50).

Fig 3. AFM (Atomic Force Microscopy) analysis result (10 μ m $\times$ 10 μ m)

Fig 4. Size distribution of polymeric nanoparticle with three measurements with ZETA sizer.

Fig 5. The size distribution of polymeric nanoparticle was analyzed at different pH ranges with ZETA sizer **A)** pH4 **B)** pH6 **C)** pH7 **D)** pH8 **E)** pH10.

Fig 6. Fourier transform infrared (FTIR) spectra of p(HEMA) (black), and L-glutamic-acid-g-p(HEMA) (red).

Fig 7. The swelling ratio of L-glutamic acid-g-p(HEMA) in distilled water and phosphate buffered solution (pH 7.4) at different time. After 15 min, the swelling ratio was fixed in both environments.

Fig 8. Cytotoxicity test results of p(HEMA) and L-glutamic acid-g-p(HEMA) at different concentration on 16HBE and THP-1 cell line at 24 h exposure time with WST-1 and LDH assay. **A.** p(HEMA) and **B.** L-glutamic acid-g-p(HEMA) effects on 16HBE cell line with WST-1 assay. **C.** p(HEMA) and **D.** L-glutamic acid-g-p(HEMA) effects on THP-1 cell line with WST-1 assay. **E.** p(HEMA) and **F.** L-glutamic acid-g-p(HEMA) effects on 16HBE cell line with LDH assay. **G.** p(HEMA) and **H.** L-glutamic acid-g-p(HEMA) effects on THP-1 cell line with LDH assay. The cell viability was calculated as a percentage from the viability of the control (untreated) cells. The viability of the control cells was considered 100%. The results are expressed as means $\pm$ SD from three repeats and three independent experiments.

Fig 9. Hemolysis rates of L-glutamic acid-g-p(HEMA) at different concentrations. The results are expressed as means $\pm$ SD from three repeats in experiment.

Fig 10. In vitro cell migration assay test results. The closure difference was calculated as 2 hours and the closure percentage was calculated as 21%.

Fig 11. A. Comet assay results scored in arbitrary units, under Olympus fluorescence microscope at a $\times 10$ magnification. Arbitrary units are expressed in 4 categories **a)** Negative Control **b)** Little damaged DNA strand fragmentation **c)** Medium damaged DNA strand fragmentation **d)** Maximum Damaged DNA strand fragmentation **e)** Positive Control; **B.** DNA damage induced by different concentration, values presented are Mean $\pm$ SD; in total 100 comets were scored vary between 0 (100 undamaged cells) and 400 (100 maximally damaged cells); **C.** Particle density on slides as a result of dye-particle binding.

Fig 12. HET-CAM test results within time points, after exposure different concentrations of L-glutamic acid-g-p(HEMA), 0.9% NaCl as negative control and 0.1N NaOH as positive control with three repeats of experiment.

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