

Synthesis, Swelling Properties and Evaluation of Genotoxicity of Hydrogels Based on (Meth)acrylates and Itaconic Acid

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In this study we prepared hydrogels based on 2-hydroxyethyl methacrylate (HEMA): PHEMA homopolymer and two terpolymers of HEMA, itaconic acid (IA) and two poly(alkylene glycol) (meth)acrylates (PAGM): poly(ethylene glycol)₆ acrylate (P(HEMA/IA/PAGM1)) and poly(propylene glycol)₅ methacrylate (P(HEMA/IA/PAGM2)). Hydrogels were synthesized by gamma-irradiated radical polymerization and subjected to swelling measurements and genotoxicity evaluation. Swelling studies confirmed that these hydrogels deserve consideration as biomaterials due to their ability to swell in phosphate buffer but maintaining physical integrity for a prolonged contact time after equilibrium state has been reached. Comet assay showed certain genotoxic effect following cell exposure to extracts of hydrogels, which was dependent on the concentration of extracts, chemical composition of hydrogels and the degree of crosslinking. The influence of concentration on genotoxicity was the most pronounced. The synthesis of these novel HEMA-based hydrogels should be optimized so as to reduce their toxicity and enable the use in clinical practice.

Keywords: HEMA-based hydrogels, itaconic acid, swelling, genotoxicity, Comet assay

1. Introduction

Hydrogels are three-dimensional crosslinked polymer networks that exhibit the ability to swell and retain a significant amount of water or biological fluids within their structure^{1,2}. In the swollen state, hydrogels are soft and rubbery, resembling natural living tissue more than any other class of synthetic biomaterials³⁻⁶. Therefore, hydrogels have found widespread applications in medicine as wound dressings^{6,7}, contact lenses⁸ and artificial skin⁹; in tissue engineering for reparation and regeneration of organs and tissues such as bones^{10,11} and cartilages¹², and in pharmacy as controlled drug delivery systems^{6,13-15}.

Hydrogels based on 2-hydroxyethyl methacrylate (HEMA) are very commonly studied for use as biomaterials in different applications because of their excellent physicochemical properties¹⁶. HEMA is generally prepared in the form of copolymeric hydrogels with ionic or more hydrophilic monomers¹⁷. Copolymers of HEMA with methacrylic¹⁸, acrylic¹⁹ and itaconic acid²⁰, as pH sensitive components, have been reported previously as stimuli-responsive hydrogels for use in drug delivery systems⁹. Although many HEMA-based hydrogels are generally considered to be non-toxic and have been used in biomedical applications²¹⁻²³, the information

on their safety is still incomplete. HEMA, as (meth)acrylate monomer, is capable to induce various adverse effects at cellular level, such as oxidative stress, cell cycle disturbance and apoptosis²⁴⁻²⁷. Table 1 shows the results of different *in vitro* studies which have demonstrated that HEMA is a potent mediator of DNA damage, at concentrations ranging from micromolar to millimolar. On the other side, only few studies tested the genotoxic potential of polymerized hydrogels, and the results were controversial.

Most of the problems associated with hydrogels regarding toxicity are unreacted compounds such as monomers, oligomers, initiators, stabilizers, inhibitors, emulsifiers and crosslinkers used in hydrogel synthesis. The process of hydrogel polymerization is almost always incomplete, resulting in the presence of unreacted compounds, which in turn can cause cytotoxic and genotoxic effects leading to irreversible disturbance of basic cellular functions¹³. Moreover, Samuelsen et al.³⁶ suggested that, if released at low concentrations for a prolonged period of time, HEMA could reduce the cellular proliferation rate and lead to apoptosis probably due to DNA damage. Since genotoxicity can limit or completely disable the use of materials in clinical practice, it is very important to evaluate potential genotoxicity of any novel material intended for implantation or long term exposure.

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Table 1: Genotoxic potential of HEMA monomer and extracts of polymerized hydrogels assessed in various cell types by employing different genotoxicity tests.

Monomer	Cell line	Genotoxicity test (monomer concentration)	Result	Reference
HEMA	V79 Chinese hamster lung fibroblasts	Micronucleus Comet assay (1-18 mM)	Genotoxic effect in a dose-dependent manner.	Lee et al. 2006 ²⁸
	V79 Chinese hamster lung fibroblasts	Micronucleus (2-8 mM)	Genotoxic effect in a dose-dependent manner.	Schweickl et al. 2007 ²⁹
	Human peripheral blood lymphocytes	Comet assay	Mild enhancement of DNA migration.	Kleinsasser et al. 2004 ³⁰
	Human samples of salivary glands	Comet assay	Significant DNA migration in both cell types.	Kleinsasser et al. 2006 ³¹
	Human peripheral blood lymphocytes			
	Human peripheral blood lymphocytes	Comet assay (0-10 mM)	Increased DNA damage in a dose-dependent manner	Pawlowska et al. 2010 ³²
	A549 lung-tumour cells	Human peripheral blood lymphocytes	Comet assay Micronucleus Chromosome aberration (CA) Sister chromatid exchange (SCE) (10 µM-1 mM)	Genotoxic effect was measurable in the comet assay at 1 mM of HEMA, but not in the micronucleus test. A significant dose-dependent increase in the frequency of CAs and SCEs could be demonstrated in all tested concentrations.
	Human gingival fibroblasts		Comet assay (1-10 mM)	Increased tail DNA in a dose-dependent manner.
IA			N/A	
PAGM			N/A	
Hydrogel	Cell line	Genotoxicity test (extract concentration)	Result	Reference
NVP-AA ^a	HepG2	Comet assay (0.25-25 mg/ml)	Four to six fold increase in DNA breaks.	Devine et al. 2006 ⁵
NVP-AA	HepG2 HaCaT	Comet assay Ames assay	Genocompatible	Kirf et al. 2010 ²³
ALPF-HEMA ^b	L929 fibroblasts	Comet assay	Genocompatible	Finosh et al. 2014 ³⁵

^a *N*-vinyl-2-pyrrolidone/acrylic acid ^b Alginate-polypropylene fumarate/2-hydroxyethyl methacrylate

Over the past decade, Comet assay was developed as a rapid, simple, and sensitive technique for analyzing and quantifying DNA damage in individual cells^{37,38}. The Comet assay, also called single cell gel electrophoresis (SCGE), combines DNA gel electrophoresis with fluorescence microscopy in order to visualize migration of DNA strands from individual agarose-embedded cells³⁹. The resulting image resembles a “comet” with a distinct head consisting of intact DNA, and a tail which contains damaged or broken pieces of DNA. The amount of DNA liberated from the head of the comet during electrophoresis depends on genotoxic potential of tested compound³⁷. Over time this method has been improved and today it is suitable for detection of DNA damage caused by double and single strand breaks, alkali labile sites, DNA crosslinking with DNA or protein and oxidative base damage. The advantages of Comet assay, relative to the other genotoxicity tests, include its high

sensitivity for detecting low levels of both single and double stranded breaks in damaged DNA, small number of cells per sample, flexibility, low cost and ease of application^{40,41}. Comet assay is increasingly used to test genotoxicity of hydrogels’ extracts⁴² and other biomaterials^{13,30}.

In our previous investigation we reported the radiation-induced synthesis of copolymeric hydrogels composed of 2-hydroxyethyl methacrylate (HEMA), itaconic acid (IA) and different poly(alkylene glycol) (meth)acrylates (PAGM)⁴³. The PAGM components and itaconic acid were used to improve the hydrophilicity of PHEMA. Itaconic acid, as a ionic component, imparts pH sensitivity and influences the swelling and mechanical properties of hydrogels²⁰. PHEMA and P(HEMA/IA/PAGM) hydrogels were characterized by Fourier transform infrared (FT-IR) spectroscopy, scanning electron microscopy (SEM) and thermogravimetric (TG) analysis. These analyses confirmed that they have an adequate

chemical structure, porosity and thermal properties to be used as multifunctional hydrogels in biomedical applications, while *in vitro* cytotoxicity test showed that none of the tested hydrogels were cytotoxic⁴³.

The aim of the present study was to further characterize these novel HEMA-based hydrogels through evaluation of their swelling properties and genotoxic potential *in vitro*. The examined hydrogels were prepared in our laboratory by gamma-irradiated free radical polymerization and included: PHEMA, poly(2-hydroxyethyl methacrylate/itaconic acid/poly(ethylene glycol)₆ acrylate (P(HEMA/IA/PAGM1)) and poly(2-hydroxyethyl methacrylate/itaconic acid/poly(propylene glycol)₃ methacrylate (P(HEMA/IA/PAGM2)). To the best of our knowledge, these are the first studies referring to the genotoxic potential of hydrogels composed of above mentioned components, evaluated by Comet assay.

2. Materials and Methods

2.1. Chemicals

2-Hydroxyethyl methacrylate (HEMA), itaconic acid (IA) (both from Sigma-Aldrich, St. Louis, MO, USA), poly(alkylene glycol) (meth)acrylates: poly(ethylene glycol)₆ acrylate (PAGM1) and poly(propylene glycol)₃ methacrylate (PAGM2) (both from Laporte Chemicals, Luton, UK) were used as components for hydrogel preparation. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), stable glutamine, antibiotic-antimycotic solution, Trypsin-EDTA solution were purchased from Biological Industries (Kibbutz Beit-Haemek, Israel). Low melting point agarose and ethidium bromide (EtBr) were obtained from SERVA (Heidelberg, Germany) while regular agarose was obtained from Applied Biosystems (Foster city, CA, USA). Trypan blue dye, disodium EDTA, Tris and Triton X-100 were obtained from Sigma-Aldrich, St. Louis, MO, USA.

2.2. Preparation of hydrogels

The PHEMA and P(HEMA/IA/PAGM) hydrogels were prepared by gamma-irradiated free radical polymerization. The feed composition for each sample is listed in Table 2. According to the PAGM component samples were designated as P(HEMA/IA/PAGM1) and P(HEMA/IA/PAGM2). The same conditions were used to prepare the PHEMA hydrogel. The reaction mixture was degassed prior to polymerization and placed between two glass plates sealed with a PVC spacer (2 mm thick). The reaction solution was irradiated in a ⁶⁰Co radiation source, under ambient conditions, at a dose rate of 0.5 kGy/h, to absorbed dose of 25 kGy. After the reaction, the hydrogels were cut into discs and immersed in water for one week. Water was changed daily and collected. After 7 days, the collected water was concentrated to a smaller volume by evaporation and was used for determination of unreacted monomers. The

amount of unreacted IA was determined by titration of extract against NaOH (0.05 mol/L) to phenolphthalein end point. On the other hand, the amount of unreacted HEMA and PAGM components was determined using UV spectroscopy⁴³.

In all cases, the processes indicate that the conversion during polymerization/crosslinking reaction was high as demonstrated in Table 2.

2.3. Swelling study

Dynamic swelling measurements were performed in phosphate buffer at pH 7.4 and temperature of 37 °C. Swollen gels were removed from the swelling medium at regular intervals, dried superficially with filter paper, weighed and placed in the same bath until constant weight was reached. The amount of fluid absorbed was monitored gravimetrically. The equilibrium degree of swelling (q_e) was calculated as follows:

$$q_e = (m_e - m_o) / m_o \quad (1)$$

where m_e is the weight of swollen hydrogel at equilibrium and m_o is the weight of xerogel^{44,45}. All swelling experiments were performed in triplicate.

The most important parameters characterizing a hydrogel network structure are the molecular weight between crosslinks (\bar{M}_c), and the effective crosslinking density (ν_e). Caykara et al.⁴⁶ described the molecular weight of the polymer chain between two neighboring crosslinks for ionic polymer networks by following relation:

$$\frac{V_1 X^2 \phi_{2,s}^2 \left(\frac{2K_{a1}K_{a2} + 10^{-pH} K_{a1}}{2(10^{-pH})^2 + 10^{-pH} K_{a1} + K_{a1}K_{a2}} \right)^2}{4I\bar{V}_r^2} = [\ln(1 - \phi_{2,s}) + \phi_{2,s} + \chi\phi_{2,s}^2] + \left(\frac{V_1\rho}{\bar{M}_c} \right) \phi_{2,r}^{2/3} \phi_{2,s}^{1/3} \quad (2)$$

where K_{a1} and K_{a2} are the first and second dissociation constants of a diprotic acid, X is the weight fraction of ionisable polymer in the system, I is ionic strength of the swelling medium, $\phi_{2,s}$ is the polymer volume fraction in the swollen gel, $\phi_{2,r}$ is the polymer volume fraction in the relaxed state, V_1 is the molar volume of water, ρ is the polymer density, \bar{V}_r is the average molar volume of polymer repeating units, and χ is the Flory polymer-solvent interaction parameter. Polymer volume fraction in the relaxed state ($\phi_{2,r}$) is determined according to the following formula:

$$(\phi_{2,r}) = V_d / V_r \quad (3)$$

where V_d is volume of the polymer sample in dry state and V_r is the volume of the polymer sample in relaxed state, immediately after synthesis. The volumes were calculated by measuring dimensions of hydrogels discs. The effective crosslinking density (ν_e) was calculated using the relation:

$$\nu_e = \rho / \bar{M}_c \quad (4)$$

Table 2: Feed compositions for P(HEMA/IA/PAGM) and PHEMA hydrogels.

Component	P(HEMA/IA/PAGM1)	P(HEMA/IA/PAGM2)	PHEMA
HEMA (mol%)	70	70	100
PAGM 1 and 2 (mol%)	28	28	0
IA (mol%)	2	2	0
HEMA+IA+PAGM (wt%)	10	10	10
Demineralized water (wt%)	45	45	45
Ethyl alcohol (wt%)	45	45	45
Conversion (wt%)	97.5	98.1	99

2.4. Preparation of hydrogels extracts

Individual hydrogel discs were weighed (0.2 g in total) and immersed in 5 ml of complete DMEM (DMEM supplemented with 10% fetal bovine serum, antimycotic-antibiotic solution and 2 mM stable glutamine). Extraction of hydrogels was performed under sterile conditions in a water bath at 37 °C during 3 days. Extracts were then discarded and diluted with complete DMEM to give final concentrations of 10% and 50%.

2.5. Cell culture

Genotoxic potential of hydrogels' extracts was tested using HeLa cell line (ATCC, Manassas, USA). Cells were grown in complete DMEM at 37 °C in humidified atmosphere containing 5% CO₂. Medium was replaced every 2–3 days. After reaching approximately 70% confluence, cells were detached by using Trypsin–EDTA solution, centrifuged at 4 °C for 10 min at 1000 rpm, washed and appropriate cell density was set up by using Trypan Blue Dye.

2.6. Treatment of cells

HeLa cells were seeded in 12-well culture plate at a density of 3×10^4 cells per ml. After 24 hours medium was replaced with extracts of hydrogels in concentrations of 10% and 50%. Incubation of cells with extracts was continued for the next 24 hours. In parallel, the control cells were treated with 200 μ M H₂O₂ for 15 min as positive control. Negative control were cells incubated only with complete DMEM (untreated cells). All samples, as well as controls were examined in triplicate.

2.7. Comet assay

Comet assay was performed according to procedure described by Dhawan et al.⁴⁷ with some modifications. Following treatment, hydrogels' extracts and control media were removed and cell viability was determined as quickly as possible by using Trypan Blue Dye Exclusion Method to avoid false positive responses due to cytotoxicity. Subsequently, 100 μ l of the cell suspension was mixed with low-melting

point agarose (0.75% wt), and 50 μ l cell-aliquots were spread on slides precoated with normal-melting point agarose (1% wt). The slides were immersed into cold, freshly made lysis solution (2.5 M NaCl, 100 mM disodium EDTA, 10 mM Tris, 1% Triton X-100; pH 10). Lysis of cells was performed in dark and cold room (5 °C) for 2 hours. Following alkaline unwinding for 40 min by immersion into cold electrophoresis buffer (300 mM NaOH, 1 mM EDTA; pH \geq 13), the slides were subjected to electrophoresis at 30 V and 300 mA for 40 min. Slides were then neutralized in 400 mM Tris buffer (pH 4) for 5 min and stained with EtBr (20 μ g/mL). DNA migration was observed by using fluorescence microscope LEICA DMR (Wetzlar, Germany) and images were taken from approximately 10 fields of the each slide by using LEICA DC 300 camera. Image analysis system CometScore v1.5 (TriTek Corp., USA) was employed to determine the percentage of DNA in comet tail (%DNA_t) and the tail moment (M_t). These parameters were calculated as follows:

$$\%DNA_t = \frac{I_t}{I_c} \times 100 \quad (5)$$

$$M_t = \%DNA_t \times L_t \quad (6)$$

where I_t is the total comet tail intensity, I_c is the total comet intensity and L_t is the tail length.

2.8. Statistical analysis

The Comet assay was conducted on duplicate slides per concentration of extract of each hydrogel, as well as controls, with approximately 100 cells scored per slide. Normality of data was evaluated with the Kolmogorov-Smirnov test. The statistical difference between control and treated cells was analyzed with the nonparametric Mann-Whitney test using %DNA_t and M_t values. The significance level was set at $p \leq 0.01$. Multiple correlation analysis was performed in order to estimate the combined influence of independent variables (extract concentration, crosslinking density (v_c) and swelling equilibrium (q_e)) on the dependent variable (%DNA_t). Regression analysis was employed to further define degree and type of relationship between independent and dependent variables, which showed significant correlation. Statistical analysis was performed using SPSS Statistics Version 17 for Windows.

3. Results and Discussion

3.1. Swelling properties

The physical behavior of hydrogels is dependent on their equilibrium and dynamic swelling behavior in aqueous media. For application of hydrogels, swelling and shrinking kinetics are very important, e.g. in controlled release drug delivery systems, where the kinetics determine the rate of diffusion of the active component from the gel matrix and in gel extraction where the gel is swollen and shrunk several times⁴⁸. Swelling kinetics of synthesized samples was determined by monitoring the swelling process in phosphate buffer mimicking physiological conditions (pH 7.4 and 37 °C). The equilibrium swelling degree (q_e) values were in the range of 0.42 - 4.28 (Figure 1). After the equilibrium swelling was reached all samples were kept in buffer solution for additional 10 days. Figure 1 shows that in that period q_e values remained practically constant. Furthermore, at the end of this period, all samples had a soft consistency and exhibited a transparent and colorless appearance.

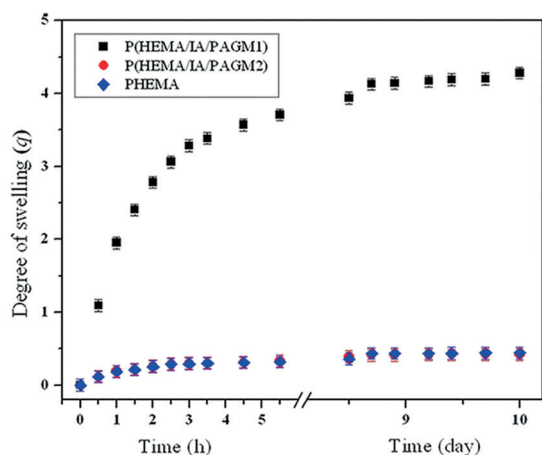


Figure 1: Swelling profiles and equilibrium degree of swelling (q_e) of HEMA-based hydrogels in phosphate buffer (pH 7.4) at 37 °C.

The swelling properties depend on many factors such as network density, solvent nature and polymer-solvent interaction parameter^{4,49}. P(HEMA/IA/PAGM1) hydrogel swells more than P(HEMA/IA/PAGM2) terpolymer and PHEMA homopolymer network. Terpolymer hydrogels contain different PAGM components, with pendent chains of different length which influenced the swelling process. The P(HEMA/IA/PAGM1) sample, with incorporated hydrophilic acrylate residue in the main chain and ethylene glycol pendant chains, reached substantially higher swelling degree than P(HEMA/IA/PAGM2) and PHEMA samples, with incorporated less hydrophilic methacrylate residues in the main chain and hydrophobic propylene glycol pendant chains in the case of P(HEMA/IA/PAGM2) sample.

According to the potential biomedical application, the calculations of crosslinking densities were done for the results obtained in pH 7.4, at 37 °C. The values of effective crosslinking densities for PHEMA, P(HEMA/IA/PAGM1) and P(HEMA/IA/PAGM2) were calculated as 48.18, 0.187 and 47.51 mol/dm³, respectively. It is evident that in case of terpolymers (P(HEMA/IA/PAGM1) and P(HEMA/IA/PAGM2)), effective crosslinking density depends very much on PAGM component, i.e. alkylene glycol pendant chains in the polymeric network. The v_e values follow the expected trend in accordance with the hydrophilic character of the PAGM component and the crosslinking degree of the sample. The sample with the highest equilibrium degree of swelling (P(HEMA/IA/PAGM1)) has the lowest v_e value. Due to the higher sensitivity of propylene glycol units in P(HEMA/IA/PAGM2) hydrogel to gamma radiation, in comparison to ethylene glycol units in P(HEMA/IA/PAGM1) hydrogel, a higher crosslinking density in the case of P(HEMA/IA/PAGM2) hydrogel was obtained. Therefore, the crosslinking density values along with the hydrophobic/hydrophilic character of monomer residues in hydrogel are in good accordance with their q_e values.

3.2. Comet assay

Table 3 represents the results of Trypan Blue Dye Exclusion Method, employed to evaluate cytotoxicity concurrently with Comet assay.

Although a dose-dependent decrease in HeLa cell viability was observed, more than 70% of cells were viable after treatment with hydrogels' extracts, regardless the hydrogel type and extract concentration. According to ISO standard (ISO 10993-12), preparation of fluid extracts of the device materials is the most appropriate technique when there is a need to determine toxicity of possible chemical leachables, especially by Comet assay^{5,38}. Because DNA damage is associated with cell death, evaluation of genotoxicity is only relevant at sub-cytotoxic concentrations of examined samples. It is crucial to evaluate cytotoxicity at the end of the exposure period and general approach is to exclude concentrations that decrease cell viability by more than 30%^{50,51}. Since the results of the Trypan Blue assay performed in this study show that cell viability, after exposure to the both concentrations of tested extracts, is higher than 70%, none of the hydrogels' extracts can be considered cytotoxic to HeLa cells, and these extracts concentrations (10% and 50%) are suitable for further genotoxicity examination. Also, cell viability determined in this study was similar with the results obtained by using neutral red method in our previous characterization of these hydrogels⁴³.

Figure 2 shows the representative images of HeLa cells after staining with EtBr in Comet assay.

The results of the Comet assay show that extracts of all tested hydrogels are capable to induce certain genotoxic

Table 3. Cell viability and Comet assay data for HeLa cells exposed to the extracts of HEMA-based hydrogels for 24 h.

Treatment	Cell viability (%)	DNA damage \pm S.E.M	
		Mean %DNA _t	Mean M _t
Untreated control	94.8	3.19 \pm 0.63	0.41 \pm 0.21
PHEMA			
10%	89.7	6.19 \pm 1.47	0.76 \pm 0.62
50%	79.4	11.03 \pm 1.39*	1.59 \pm 0.53*
P(HEMA/IA/PAGM1)			
10%	88.1	7.47 \pm 1.8	1.53 \pm 0.42
50%	76.6	25.39 \pm 4.62*	8.78 \pm 1.35*
P(HEMA/IA/PAGM2)			
10%	87.8	6.57 \pm 0.94	0.71 \pm 0.26
50%	74.7	23.82 \pm 2.15*	4.63 \pm 0.85*
Positive control ^a	69.5	21.11 \pm 1.85*	8.59 \pm 1.71*

^a Positive control agent: 200 μ M H₂O₂; * Denotes a significant difference from the untreated control ($p \leq 0.01$).

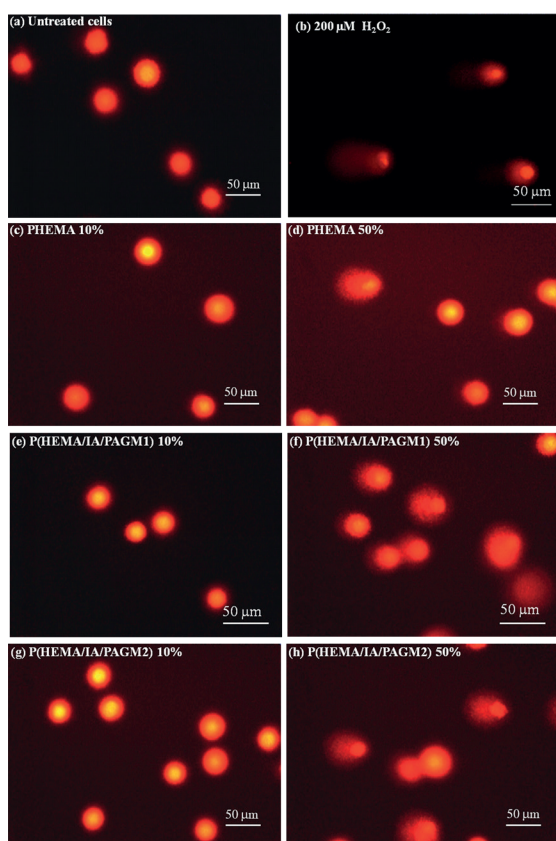


Figure 2: Representative images of HeLa cells in Comet assay; untreated cells (a), cells treated with 200 μ M H₂O₂ (b) and cells treated with two concentrations (10% and 50%) of PHEMA (c, d), P(HEMA/IA/PAGM1) (e, f) and P(HEMA/IA/PAGM2) (g, h) hydrogels' extracts. Magnification $\times 400$.

effects, as measured by %DNA_t and M_t parameters (Table 3, Figure 2). Extracts of tested HEMA-based hydrogels in concentration of 10% induced no significant increase in %DNA_t and M_t compared with untreated cells. Significant augmentation of DNA migration ($p < 0.01$) was evident only

after treatment of HeLa cells with higher extract concentration (50%). Extracts of PHEMA, P(HEMA/IA/PAGM1) and P(HEMA/IA/PAGM2) hydrogels in concentration of 50% induced 1.8 ($p \leq 0.05$), 3.4 ($p \leq 0.01$) and 3.6 ($p \leq 0.01$) times higher %DNA_t compared with %DNA_t induced by lower concentration (10%) of the same extracts. Also, higher extracts' concentrations induced up to 3.6 times higher M_t than same extracts of lower concentration. M_t parameter is also presented as Tukey box plot diagram (Figure 3). The outliers that can be seen in untreated control as well as in treated cells, are probably the cells that underwent spontaneous apoptosis/necrosis events²⁹ and were not included in the analysis.

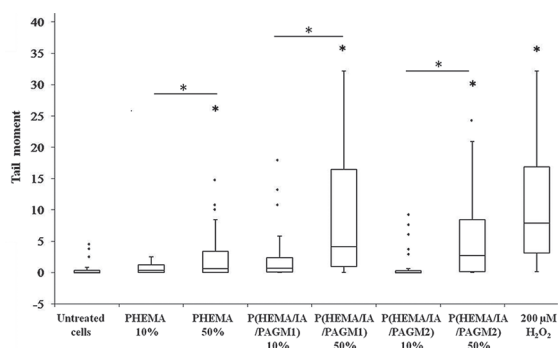


Figure 3: Tukey box plot diagram for tail moment parameter of HeLa cells treated with 10% and 50% extracts of HEMA-based hydrogels, 200 μ M H₂O₂ as positive control and untreated cells. Whiskers extend to the 1.5 box heights. If minimum and maximum values are out of this range, then they are shown as outliers (dots above boxes). *Denotes a statistically significant differences between concentrations of extracts and compared with untreated control ($p \leq 0.01$).

The extent of DNA damage depends on extract concentration as well as on properties of hydrogel such as crosslinking density (ν_c) and swelling equilibrium (q_d). In order to analyze the impact of each independent variable

(extract concentration, v_e and q_e) on the dependent variable (%DNA_i), we performed multiple correlation analysis.

As presented in Figure 4a, extract concentration showed the strongest positive impact on genotoxicity as determined by correlation coefficient (0.82). We further analyzed the relationship between extract concentration and genotoxicity by employing regression analysis. As can be seen in Figure 4b, coefficient of determination (R^2) equals 0.68, which means that 68% of the variation in genotoxicity is explained by the extract concentration. According to the coefficients, the equation of regression line is:

$$y = 3.41 + 0.33x \quad (7)$$

In other words, for each unit increase in extract concentration (x), genotoxicity (y) increases with 0.33 units. This could be valuable information when there is a need to predict the genotoxic effect of different concentrations of extract.

The %DNA_i values of the cells treated with the same concentration (50%) of extracts of tested hydrogels were compared. The results indicate that genotoxic potential increases in this order: PHEMA < P(HEMA/IA/PAGM2) < P(HEMA/IA/PAGM1). In other words, extracts of P(HEMA/IA/PAGM1) and P(HEMA/IA/PAGM2) hydrogels induced about 2.2 ($p \leq 0.01$) times higher %DNA_i than PHEMA hydrogel's

extract. The extent of DNA damage induced by P(HEMA/IA/PAGM1) is about 1.1 ($p \leq 0.05$) time higher than in the case of P(HEMA/IA/PAGM2) hydrogels' extract. Therefore, it is obvious that properties of hydrogel such as its degree of crosslinking, swelling equilibrium and genotoxic potential, are related. Impact of these independent variables on genotoxicity was also tested by employing multiple correlation analysis (Figure 4a). Crosslinking density of the hydrogel and its swelling equilibrium are in almost perfect negative correlation - increasing of crosslinking density reduces the swelling equilibrium. These two variables have opposite influence on genotoxicity (%DNA_i) - increasing of crosslinking density reduces while increasing of swelling equilibrium increases the genotoxic effect of hydrogel extracts. However, the influence of crosslinking density and swelling equilibrium on overall genotoxicity is relatively weak as measured by correlation coefficients (≈ 0.26 , Figure 4a). PHEMA homopolymer and P(HEMA/IA/PAGM2) terpolymer have higher effective crosslinking density (v_e), lower equilibrium degree of swelling (q_e), and show less pronounced genotoxic effect than P(HEMA/IA/PAGM1) terpolymer. The generally accepted property of highly crosslinked polymers is that they are more resistant to degradative processes and elution of unreacted components, based on more limited space and pathways available for solvent molecules to diffuse within

(a)			
	Extract concentration	Crosslinking density (v_e)	Swelling equilibrium (q_e)
Extract concentration	1	-	-
Crosslinking density (v_e)	0	1	-
Swelling equilibrium (q_e)	0	-0.9997	1
Genotoxicity (%DNA _i)	0.82	-0.27	0.26

(b)	
Multiple R	0.82
R^2	0.68
Significance F	0.04
Coefficients	
Genotoxicity (y)	3.41
Extract concentration (x)	0.33

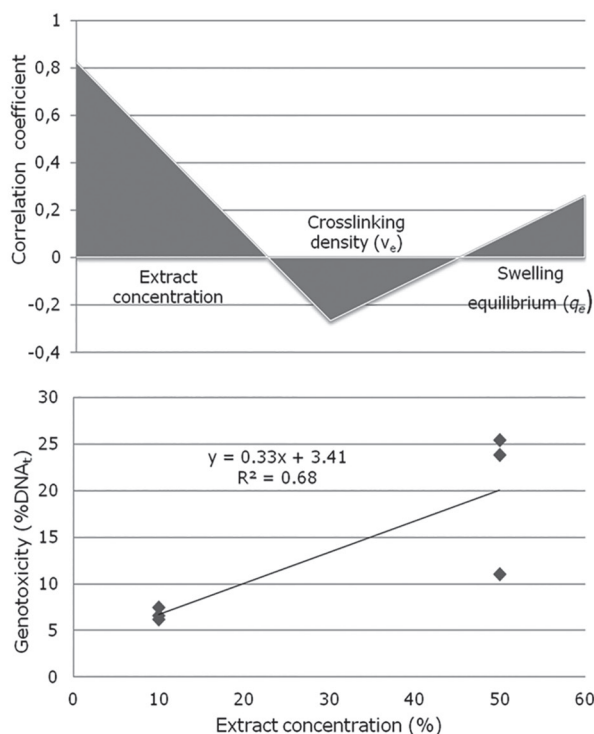


Figure 4: (a) Multiple correlation analysis employed to determine the strength and direction of the association between the independent variables (extract concentration, crosslinking density (v_e) and swelling equilibrium (q_e)) and the one dependent variable (%DNA_i). Correlation coefficient ranges between -1 to +1, and quantifies the strength (the closer coefficient is to 1, the stronger linear association is) and the direction (positive or negative) of the association. (b) Regression analysis of the relationship between extract concentration and genotoxicity, based on significant correlation among these two variables.

structure^{13,52,53}. The elution of unreacted components from hydrogels is influenced by several factors including the chemical composition of components in hydrogel synthesis, the conversion during polymerization reaction and the degree of crosslinking of the polymeric network¹³. Due to the lowest degree of crosslinking, extract of P(HEMA/IA/PAGM1), which showed the highest level of genotoxicity, probably contains more unreacted components comparing with extracts of PHEMA and P(HEMA/IA/PAGM2) samples. In the case of tested hydrogels, these unreacted monomers are IA, methacrylates HEMA and PAGM2, and acrylate PAGM1. It is well known that unreacted monomers in hydrogels can cause the living tissue damage^{13,54}. Itaconic acid is a component of natural origin and it is supposed that will not induce genotoxicity. (Meth)acrylate monomers are reported in the literature to exhibit genotoxic effects²⁴⁻³⁰. Furthermore, Dearfield et al.⁵⁵ showed that acrylates are generally more potent to induce mutations, aberrations and micronuclei than methacrylates, although this appears to be structure-related (dependent upon number of functional vinyl groups and the length of oxyethylene chains). PAGM1 has functional vinyl group and longer oxyethylene chain than PAGM2 and this structural difference may be responsible for its more potent genotoxic effect detected in Comet assay. However, this study is the first step in biocompatibility assessment of these novel HEMA-based hydrogels and it is early to comment on the mechanisms underlying the increased DNA migration observed in the alkaline version of Comet assay.

4. Conclusion

Swelling studies confirmed that hydrogels based on 2-hydroxyethyl methacrylate, poly(alkylene glycol) (meth)acrylates and itaconic acid swell in phosphate buffer but maintain physical integrity and have soft and rubbery consistency even when the swelling experiments were conducted for a long time after the equilibrium state was reached. The results of the Comet assay showed that extracts of all tested hydrogels are capable to induce certain genotoxic effects, which depended on chemical composition, extract concentration as well as on degree of crosslinking of examined hydrogels. Future research would be directed toward optimization of the synthesis of these novel HEMA-based hydrogels in order to obtain hydrogels that are not genotoxic at all and, as such, may have application in clinical practice.

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6. Conflict of interests

Authors declare that they have no conflict of interests.

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