

# SYNTHESIS OF CHITOSAN AND CARBOXYMETHYL CHITOSAN HYDROGELS BY ELECTRON BEAM IRRADIATION

Renata Czechowska-Biskup, Radosław A. Wach\*, Piotr Stojek, Martyna Kamińska, Janusz M. Rosiak, Piotr Ulański

*Institute of Applied Radiation Chemistry, Faculty of Chemistry  
Lodz University of Technology,  
Wroblewskiego 15, 93-590 Lodz, Poland.  
e-mail: wach@mitr.p.lodz.pl*

## **Abstract**

*Solutions of chitosan (CS) and carboxymethyl chitosan (CMCS) were subjected to irradiation by electron beam in presence of a crosslinking agent (poly(ethylene glycol) diacrylate - PEGDA) in order to produce carboxymethyl chitosan- and chitosan-based hydrogels. PEGDA macromonomer itself undergoes simultaneous polymerization and crosslinking either in neutral water or in acidic medium. Acidic solutions of chitosan of 0.5, 1 and 2% can be effectively crosslinked with PEGDA to form a gel. Although CMCS undergoes radiation-initiated crosslinking only at high concentration in water (over 10%), the presence of PEGDA in solution facilitated hydrogel formation even at lower concentration of CMCS. Formation of CS and CMCS hydrogels required irradiation doses lower than those needed for sterilization, i.e. 25 kGy, in some cases even as low as 200 Gy. Sol-gel analysis revealed relatively high gel fraction of obtained hydrogels, up to 80%, and good swelling ability. Both parameters can be easily controlled by composition of the initial solution and irradiation dose. Possible mechanisms of crosslinking reactions were proposed, involving addition of the polysaccharide macroradicals to a terminal double bond of PEGDA.*

**Key words:** hydrogel, chitosan, carboxymethyl chitosan, radiation crosslinking, PEGDA

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## **1. Introduction**

Hydrogel dressings are applied to cure burns, pressure sores, ulcers, surgical wounds and other skin lesions. They isolate the wound from external environment, constitute a barrier for microorganisms, but are permeable to oxygen. They provide a humid environment conducive to wound healing and prevent loss of body fluids. Hydrogels have good adhesion to the wound, but lower than for intact skin, making their removal from the wound painless. These benefits result from hydrogels three-dimensional structures of mutually linked polymer chains filled with water or water-based solvents. A leading industrially implemented technology allowing to form and sterilize hydrogel dressings in single step is based on the use of ionizing radiation. Applying radiation to aqueous solutions of selected polymers allows for simultaneous cross-linking (hydrogel formation) and sterilization. So far, this method is mainly used in manufacturing hydrogel dressings based on biocompatible synthetic polymers [1].

A few approaches have been also reported to radiation crosslinking of polysaccharides. These include irradiation of a polysaccharide in the presence of regular crosslinkers of multifunctional molecules, e.g. glycidyl acrylate [2]. Other proposed methods, e.g. irradiation of a solid polysaccharide in a presence of an alkyne gas that mediates in the crosslinking reactions of a polysaccharide [3], or irradiation of a polysaccharide in solution mixture of water, alcohol and carbon tetrachloride (carbon atoms of  $\text{CCl}_4$  participate in crosslinking bond) [4] have limited applicability since the processing results in colloid or microgel structure, not a macroscopic hydrogel suitable for application as a self-standing wound dressing. A promising direction of studies on radiation-induced cross-linking of oligo- and polysaccharides emerges from the works of Coqueret et al., where particular additives, mainly low-molecular-weight aromatic compounds, allow to shift the balance of degradation to cross-linking in the direction of the latter process in certain polysaccharide blends [5-7].

Macroscopic networks – “wall-to-wall” hydrogels – can be manufactured by radiation-initiated crosslinking of water-soluble derivatives of cellulose, alginate and other polysaccharides only when highly concentrated solution of the polysaccharide, in so-called paste-like state, is irradiated, preferably at high dose rate, as obtained using an electron accelerator [8,9]. It was ascertained that high degree of substitution of a polysaccharide, with side groups having secondary or tertiary carbon atoms able to form a carbon-centered radical through abstraction of a hydrogen atom, is advantageous for crosslinking [10,11]. Particular attention was drawn to carboxylated cellulose that could be crosslinked with high yield by irradiation either with low dose rate or at the concentration as low as 0.5 - 2%. This was controlled by presence of long-lived radicals stabilized by its polyionic nature and high degree of substitution [12,13].

Chitosan (CS) is derived from chitin and for many years it has been widely used in medicine. It is soluble only in acidic conditions, whereas its derivative, carboxymethyl chitosan (CMCS) maintains advantageous biological properties of its parent biopolymer but dissolves also in aqueous solution of neutral and basic pH [14]. CMCS is characterized by a degree of substitution (DS) of hydrogen atoms in hydroxyl groups at C3 and C6 of glucosamine units by a carboxymethyl moiety (an exemplary structure of CMCS with DS = 1 can be seen in reactions 8 and 9). These two polysaccharides with proven antimicrobial activity and suitable molecular weight can be supportive in accelerating healing of wounds if they constitute hydrogel dressings.

Formation of CS or CMCS hydrogels is not straightforward - radiation treatment of chitosan and its derivatives in solid state or in a dilute aqueous solution results in their degradation, that is normally observed effect for polysaccharides. This can be utilized to

control molecular weight of chitosan for applications where specific molecular weight is necessary, particularly to induce biological effects, such as bactericidal, plant growth promotion or fat binding [14-17]. The opposite effect of radiation, i.e. crosslinking, is desirable in order to fabricate hydrogels. Crosslinking of CS induced by radiation in the absence of crosslinking agents, either in solid state or in solution, has never been achieved. Yet, carboxymethyl chitosan is prone to form hydrogels when irradiated at very high concentration (10-30%) [18,19]. Gel fraction, i.e. the insoluble part, of up to 50% can be obtained at the irradiation dose of 25 kGy. This approach allows to crosslink pure CMCS, with no additives; but, on the other hand, the initial paste is difficult to formulate homogeneously and handle.

The aim of the current study was to evaluate the possibility to form hydrogels from chitosan and alternatively carboxymethyl chitosan, both at relatively low concentration, by cross-linking initiated by ionizing radiation, with support of a crosslinking agent, the macromonomer of poly(ethylene glycol) diacrylate of moderate molecular weight. The latter substrate was chosen because of its superb efficiency in radiation-induced gel formation, even at very low doses such as applied in 3D gel dosimetry for radiotherapy [20].

## **2. Materials and Methods**

### **2.1. Chemicals/Materials**

Medical grade chitosan (CS) was obtained from Hepe Medical Chitosan GmbH (Germany). Weight-average molecular weight of CS was 200-500 kDa and the deacetylation degree was 90% as stated by the manufacturer. Carboxymethyl chitosan sodium salt (CMCS) was purchased from Kraeber & Co GmbH, its degree of deacetylation (DD) = 93.8%, degree of substitution (DS) = 96% and intrinsic viscosity in 0.1 mol dm<sup>-3</sup> NaCl was  $\eta = 2.77 \text{ dm}^3 \text{ g}^{-1}$ . Poly(ethylene glycol) diacrylate (PEGDA),  $M_w = 700 \text{ g mol}^{-1}$ , of high purity grade, was purchased from Sigma-Aldrich. All other chemicals were of analytical grade, purchased from Sigma-Aldrich. High-purity water (0.055  $\mu\text{S cm}^{-1}$ , TKA MicroPure system) was used in all experiments.

### **2.2. Preparation of solutions**

PEGDA solutions were prepared by dissolving appropriate volume of PEGDA ( $d = 1.12 \text{ g cm}^{-3}$ ) in water, stirring at room temperature for 24 h and making up with water to defined volume. Solutions of PEGDA in acetic acid were obtained by addition the appropriate volume of PEGDA, 1 mol dm<sup>-3</sup> acetic acid and water to a volumetric flask and overnight stirring. Final pH was ca. 3.

CS solutions comprising 5% of PEGDA were prepared as follows. Chitosan was dissolved in 2% acetic acid and stirred for three days. Then appropriate volumes of PEGDA and 80% acetic acid were added up to pH = 3 and the resulting solution was further stirred overnight.

Solutions of CMCS with the crosslinker (PEGDA) were prepared by dissolution of appropriate amount of CMCS in 0.1 mol dm<sup>-3</sup> solution of NaClO<sub>4</sub>. The role of perchlorate, chosen as a radiation-resistant salt, was to reduce mutual repulsive ionic interactions in CMCS. After complete dissolution of the polymer the appropriate amount of crosslinking agent was added, solution was made up with salt solution to the defined volume and stirred overnight.

### **2.3. Irradiation of samples**

Glass ampoules were filled with 5 cm<sup>3</sup> of polymer solution, saturated with argon for at least 20 min in order to remove dissolved oxygen and tightly sealed by Parafilm<sup>®</sup>. Samples were irradiated at room temperature with 6 MeV electron beam (EB) from a linear

accelerator (Elektronika ELU-6e, Russia). Average dose rate was ca. 5 kGy·min<sup>-1</sup> as determined by alanine dosimetry. The applied doses were in the range 0.2 – 25 and 0.2 – 100 kGy for chitosan and carboxymethyl chitosan solutions, respectively.

#### 2.4. Sol-gel analysis

Formed hydrogels were placed in beakers and underwent swelling in 100 cm<sup>3</sup> of water for PEGDA and carboxymethyl chitosan hydrogels or alternatively of 2% acetic acid aqueous solution for chitosan hydrogels. After several days, all hydrogels were swollen in water for the next five days. Solvents were changed daily. In the next step hydrogels were separated from sol with a sieve and weighted for calculation of the equilibrium degree of swelling (water uptake) by Eq. 1. Eventually, hydrogels were dried in an oven at 40°C, initially at atmospheric pressure and afterwards in vacuum and weighted for calculation of gel fraction by Eq. 2.

$$EDS = \frac{W_S - W_D}{W_D} \quad (1)$$

$$Gel\ fraction = \frac{W_D}{W_I} \quad (2)$$

$W_S$  represents the weight of hydrogel in its equilibrium degree of swelling in water or aqueous solution,  $W_D$  is the weight of dried hydrogel after extraction of sol part (i.e. the weight of a dry polymer network) and  $W_I$  is the mass of initially used substrates: a polysaccharide and PEGDA.

#### 2.5. FTIR spectroscopy

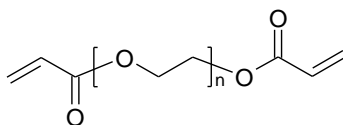
The infrared spectroscopy was used to elucidate the structure of chitosan, carboxymethyl chitosan, PEGDA and hydrogels formed after irradiation. The hydrogels were dried after extraction of soluble part. Analyses of functional groups of constituents were done by FTIR using Thermo Nicolet Avatar 330 FTIR spectrometer. Samples were prepared by mixing 1-5 mg of the dried analyte with 100-fold amount of KBr and the mixtures were compacted to form a disk. 32 scan were performed for each spectra, they were recorded in the transmission mode between 4000 and 400 cm<sup>-1</sup> and the resolution was 2 cm<sup>-1</sup>.

### 3. Results and Discussion

#### 3.1. Hydrogels of poly(ethylene glycol) diacrylate

Chitosan and its derivatives can be crosslinked to form a network by a chemical method using a crosslinking agent, for instance glutaraldehyde, genipin or tripolyphosphate [21-24]. Such method of hydrogel synthesis typically requires a complex procedure involving presence of a catalyst, thermal initiation and purification of the product. Therefore, employment of radiation to initiate crosslinking and form a gel, either with or without a crosslinking agent, in principle, is less complicated, more efficient and quick. Radiation processing utilized to obtain a network structure lasts for only seconds or minutes. Nevertheless, achieving crosslinking of CS or CMCS in aqueous solutions of moderate concentration (<10%) requires addition of a crosslinking agent. Poly(ethylene glycol) diacrylate [Fig.1] itself polymerizes and crosslinks to form a network when irradiated with relatively low doses in aqueous solution [25,26]. It was selected as a crosslinker in this study due to its low toxicity and well-known ability to form interchain linkages through its terminal double bonds. Thus, while both CS and CMCS degrade readily upon irradiation in dilute (0.5 – 5%) aqueous solutions, it was expected that presence of PEGDA (up to 5%) may change the outcome of radiation processing of these polysaccharides. Even if the ability

of poly(ethylene glycol) diacrylate to form gels through radiation processing is well known, it was important to examine the influence of particular synthesis conditions used in this work on the formation of PEGDA hydrogels.



**Figure 1.** Structure of poly(ethylene glycol) diacrylate.

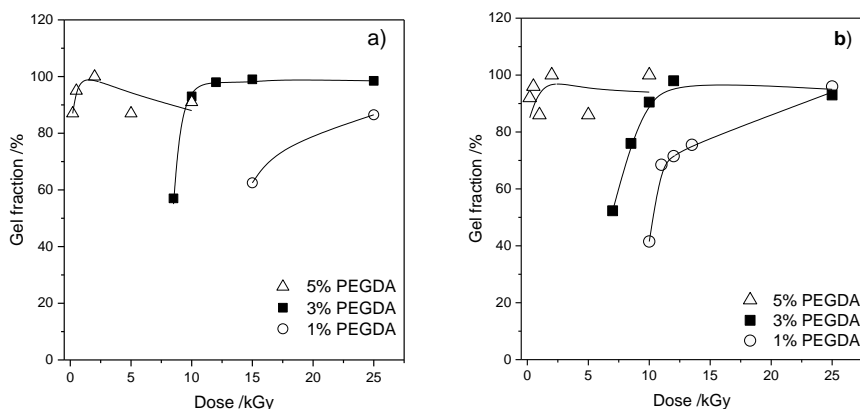
Results regarding gel formation capability of 5% aqueous solution of PEGDA at neutral pH upon EB irradiation with doses in the range of 0.2 – 10 kGy is presented in Tab. 1 and subsequently determined gel fraction values are shown in Fig. 2a. Gel fraction in hydrogels manufactured from 5% solution reached nearly 100% for all applied doses what suggests that virtually all polymer present in the sample had crosslinked, and since the dose as low as 200 Gy resulted in nearly maximum gel fraction, the crosslinking occurs with high yield. Different results were obtained for PEGDA aqueous solutions of lower concentration. No gel formation was observed at lower irradiation doses, below 5 kGy for 3% and 1% PEGDA solutions; the gel appeared after irradiation with 7.5 and 10 kGy, respectively. The gelation dose (i.e. the dose at which first macroscopic insoluble fraction can be isolated) of similar system employing argon-saturated 20% aqueous solution of PEGDA ( $M_w = 700 \text{ g mol}^{-1}$ ) was determined to be as low as 20 Gy and gel fraction rose up to 100% at 180 Gy, thus the currently obtained results are in line with that shown by Kozicki et al. [20].

**Table 1.** Gel formation in argon-saturated 1%, 3% and 5% PEGDA aqueous solutions (X - gel present, - - gel absent, nd - not examined).

PEGDA SOLUTIONS	DOSE/kGy								
	0.2	0.5	1	2	5	7.5	10	15	25
5%, pH=7	X	X	X	X	X	nd	X	nd	nd
5%, pH=3*	X	X	X	X	X	nd	X	nd	nd
3%, pH=7	-	-	-	-	-	X	X	X	X
3%, pH=3*	-	-	-	-	-	X	X	X	X
1%, pH=7	-	-	-	-	-	-	X	X	X
1%, pH=3*	-	-	-	-	-	-	X	X	X

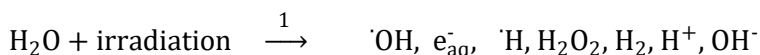
\* in acetic acid

In the view of utilization of PEGDA as a crosslinking agent, its crosslinking was evaluated in acetic acid solutions of pH 3 since chitosan is soluble only in acidic environment. Data in Tab.1 show no differences in gel formation for PEGDA solutions irradiated in neutral and acidic pH, similarly nearly no influence of the solution pH on the gel fraction could be observed in Fig. 2b. The dose required to obtain the same gel fraction are only slightly lower for PEGDA irradiated in acetic acid.

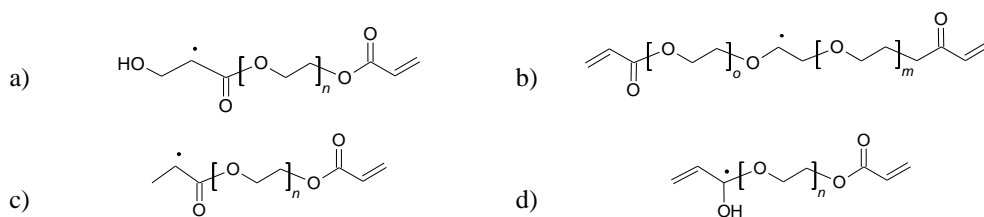


**Figure 2.** Gel fraction vs dose for PEGDA solution in a) water, b) aqueous acetic acid solution, pH = 3; saturated with argon.

Crosslinking of PEGDA involves of radicals formed at this molecule which occurs mainly through transient products of water radiolytic decomposition (reaction 1):

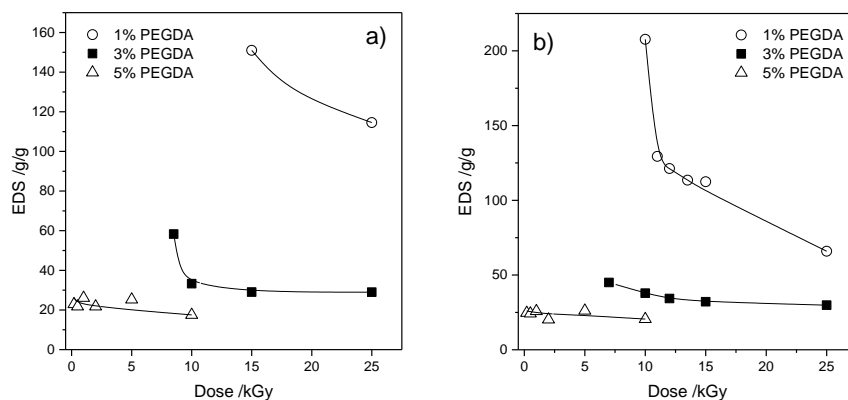


Especially hydrated electrons and hydroxyl radicals created with high yield. Both of them are highly reactive towards PEGDA - rate constants of reactions with PEGDA for hydrated electrons and hydroxyl radicals are  $1.7 \cdot 10^{10}$  and  $3.0 \cdot 10^{10} \text{ dm}^{-3} \text{ mol}^{-1} \text{ s}^{-1}$ , respectively [25]. The latter species (also hydrogen atoms, but they are formed with ten-fold smaller yield) abstract hydrogen atoms from carbon of the ethylene oxide spacer chain or, even with higher probability, add to C=C double bonds. As result, carbon-centered radicals are formed as shown in Fig. 3. a) and b). Hydrated electron addition to carbonyl carbon atom yields radical anion which at high pH undergoes relatively slow protonation with breaking the double bond giving carbon-centred radical c) in Fig. 3, or alternatively at low pH after fast protonation the radical d) persists at the carbonyl oxygen atom [27]. Taking under account that yields of reactions of water radiolysis transient products, at currently used concentrations of substrates, with PEGDA at neutral pH and pH 3 are nearly the same, it may be assumed that observed slightly different crosslinking efficiency is due to different ratio of radicals c) and d) at neutral and acidic pH, respectively.



**Figure 3.** Radicals formed at PEGDA.

Equilibrium degree of swelling in water [Fig. 4] of all hydrogels formed from 5% PEGDA solutions is ca. 20 – 25 g/g. This value indicates relatively high density of crosslinking which stabilizes the lattice and hinders its expansion. In solutions of 3% and 1% PEGDA the swelling ratio is higher at lower doses, over 40 and 100 g/g respectively, what implies lower crosslinking density. Results follow the classical dependence for radiation-formed hydrogels – EDS decreases with increasing dose [10]. The data show that irradiation of the crosslinking agent in aqueous solution at low pH results in values of the gel fraction and EDS comparable to hydrogels formed at neutral pH. Crosslinking of PEGDA in solutions acidified with acetic acid is possible, which is critical for its application as an additive in formation of hydrogels by irradiation of chitosan solutions.



**Figure 4.** Swelling of PEGDA hydrogels manufactured by EB irradiation in argon-saturated solutions of a) water, b) aqueous acetic acid solution, pH = 3, as a function of dose.

### 3.2 Chitosan and Carboxymethyl chitosan hydrogels

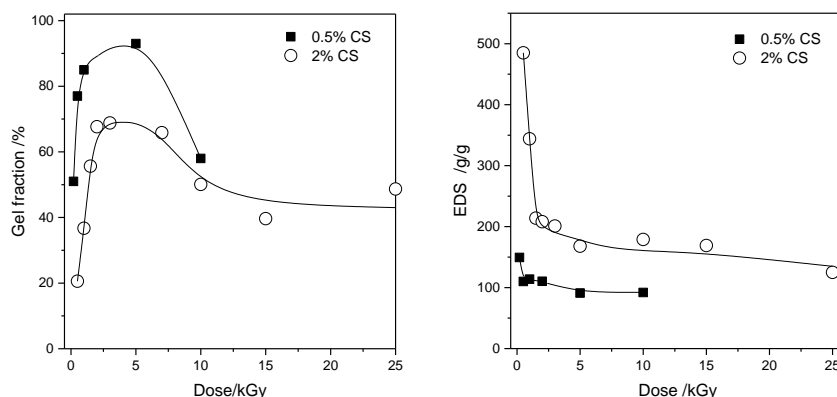
Based on the above results, concentrations of PEGDA were chosen for experiments with chitosan and carboxymethyl chitosan. The concentration of CS was initially set at 1% whereas concentration of PEGDA solutions were 1, 3 and 5% in order to evaluate the lowest amount of PEGDA where hydrogel can be obtained by irradiation. After irradiation of acidic aqueous solutions of CS with the crosslinker, gels were formed only at certain formulations. The results are presented in Tab. 2. 1% chitosan solution does not form gel in the presence of 1% PEGDA, whereas 3% PEGDA yields gels only at the highest applied doses of 25 and 30 kGy. Gels could be easily achieved even at low doses in solutions containing 5% PEGDA, also when chitosan content was 0.5 and 2%.

The sol-gel analysis of obtained hydrogels indicates the effect of CS concentration in irradiated solutions. Gel fractions and EDS of CS/PEGDA hydrogels are presented in Fig. 5. In solution of chitosan, radicals are formed both at CS backbone and PEGDA, resulting in more possible reaction pathways than in solutions of a single reagent. The highest degree of crosslinking responsible for relatively low swelling ratio of hydrogels is observed for initial solutions of 0.5% chitosan with 5% PEGDA. In this system crosslinking reactions between radicals on PEGDA chains, regardless of involving chitosan macromolecules or not, are the most efficient. In 2% chitosan solutions, the inhibition effect of this polysaccharide on the gel formation is more pronounced, leading to lower amount of gel and a higher swelling ratio. PEGDA itself, irradiated in 5% acidic water solution, formed nearly 100% of insoluble fraction at doses as low as 1-2 kGy, whereas maximum gel fraction, ca. 95 and 70% was

**Table 2.** Gel presence after irradiation of chitosan solutions with PEGDA at pH=3, (X - gel present, - - gel absent, nd - not examined)

COMPOSITION		DOSE /kGy								
CS /%	PEGDA /%	0.2	0.5	1	2	5	10	15	25	30
0.5	5	X	X	X	X	X	X	nd	nd	nd
1	1	-	-	-	-	-	-	nd	nd	-
1	3	-	-	-	-	-	-	-	X	X
1	5	X	X	X	X	X	X	nd	nd	nd
2	5	-	X	X	X	X	X	X	X	nd

obtained at doses of 2-5 kGy, for respectively 0.5 and 2% CS solutions containing 5% PEGDA. At higher doses some decrease in gel fraction of CS containing systems was observed. This may be due to the fact that after a certain dose the amount of unreacted PEGDA is already low, so the crosslinking becomes less efficient, while on the other hand there is some degradation (chain scission) in chitosan, thus the net effect may be that some of the already formed gel is partially destroyed. Swelling ability of the CS hydrogels crosslinked with PEGDA, as presented in Fig. 5b, is dependent on the applied dose. EDS of up to 150 and 500 g/g were determined for gels formed by irradiation with about 500 Gy of solutions containing 0.5 and 2% CS, respectively. Water uptake of 5% PEGDA solutions was not higher than 25 g/g at dose range 0.2 – 10 kGy. Such high EDS of CS hydrogels can be straightforwardly reduced to below 100 and 200 g/g, respectively by further irradiation with doses over 5 kGy.

**Figure 5.** Gel fraction a) and EDS in water b) of chitosan hydrogels obtained through irradiation of its aqueous acetic acid solution at pH=3, saturated by argon in the presence of 5% PEGDA.

The other polysaccharide which we attempted to crosslink was carboxymethyl chitosan. The inorganic salt in the CMCS solutions was intended to reduce mutual repulsive ionic interactions in this amphiphilic polysaccharide, and perchlorate does not participate in



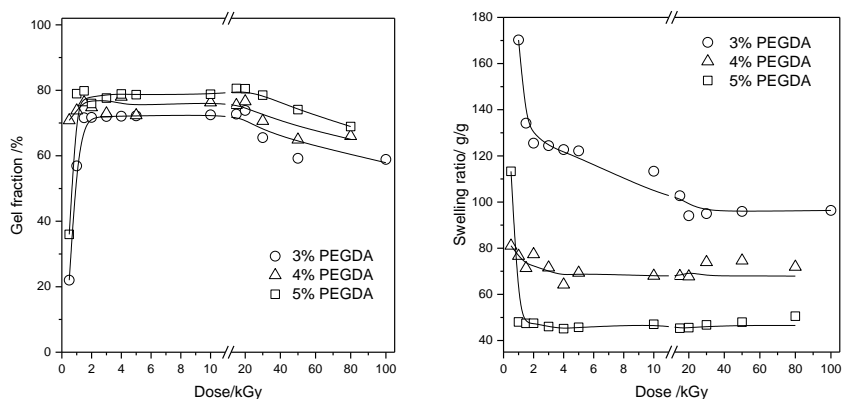
reactions with transient water radiolysis products. When irradiated in concentrated aqueous solution, over 10%, CMCS undergoes simultaneous crosslinking and degradation, through scission of glycosidic bonds, but eventually a gel part can be isolated [19]. Nevertheless, the gel fraction does not exceed 50%. Presence of a crosslinking agent is required in order to facilitate gel formation at reduced concentration of the polysaccharide. In systems containing 5% CMCS and below 3% of PEGDA degradation was still the dominant process, however at solutions containing 3%, 4%, and 5% PEGDA crosslinking predominates, as it is presented in Tab.3.

Maximum gel fraction of CMCS hydrogels crosslinked at presence of PEGDA is relatively high, in the range of 70 - 80% as shown in Fig. 6a. A slightly higher value of the gel fraction of gels manufactured from solutions containing higher amount of PEGDA is due to higher number of crosslinking bonds provided by this crosslinker. It can be seen that, as in the case of CS/PEGDA described above, also here amount of the gel somewhat decreasing for doses over 20 kGy, which is related to domination of degradation. The effect is often observed during radiation processing of polysaccharides [11].

Fig. 6b. presents the effect of irradiation dose on the water uptake capacity of CMCS hydrogels. Water absorption capacity of hydrogels is closely related to the degree of crosslinking, higher degree of crosslinking causes reduction in water uptake capacity. Therefore, for gels formed by radiation technique, the dose increase results in lowering EDS, at least in a range where crosslinking is the dominant process. This occurs for CMCS hydrogels manufactured with low doses of a few kGy. The highest values of EDS were observed for hydrogels consisting of CMCS 5% and 3% PEGDA, up to 160 g/g at the lowest applied dose. Increasing the PEGDA concentration resulted in lowering EDS. At higher doses of few tens of kGy the EDS were ca. 100, 70 and 50 g/g for 3, 4, and 5% of PEGDA, respectively. Thus, presented approach, i.e. radiation processing, offering a possibility to obtain a desired water sorption capacity, can be easily adjusted to individual needs by selecting composition of the initial mixture and appropriate dose of radiation.

**Table 3.** Gel presence after irradiation of argon-saturated 5% aqueous solution of carboxymethyl chitosan solutions with PEGDA at natural pH, in the presence of 0.1 mol dm<sup>-3</sup> NaClO<sub>4</sub> (X - gel present, - - gel absent, nd - not examined).

PEGDA SOLUTIONS	DOSE /kGy												
	0.5	1	1.5	2	3	4	5	10	15	20	30	50	100
1%	-	-	-	-	-	-	-	-	-	-	-	-	-
2%	-	-	-	-	-	-	-	-	-	-	-	-	-
3%	X	X	X	X	X	X	X	X	X	X	X	X	X
4%	X	X	X	X	X	X	X	X	X	X	X	X	nd
5%	X	X	X	X	X	X	X	X	X	X	X	X	nd

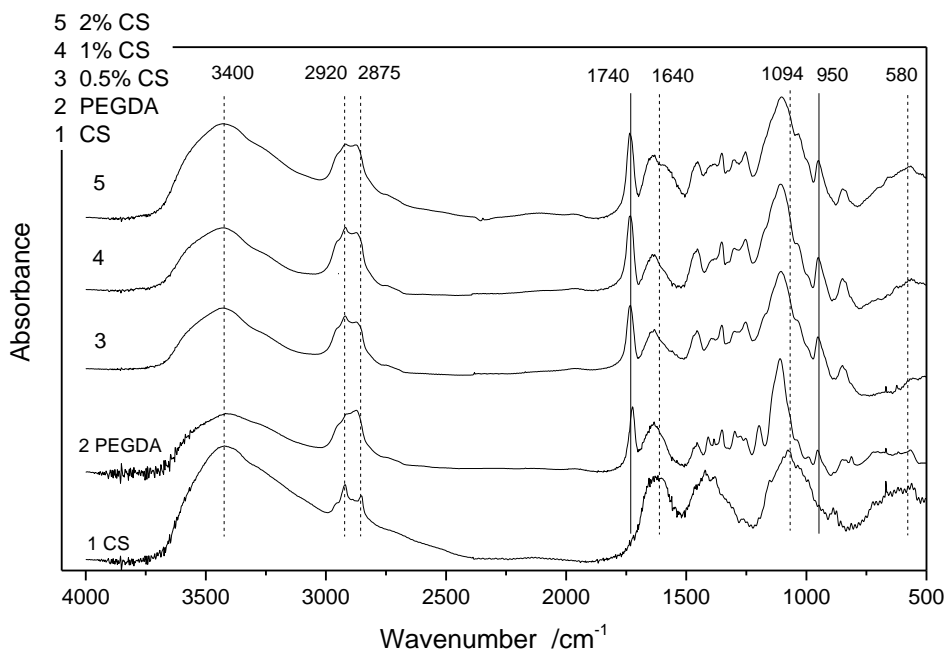


**Figure 6.** Gel fraction a) and EDS in water b) of CMCS hydrogels obtained by irradiation of its 5% aqueous solutions containing  $0.1 \text{ mol dm}^{-3} \text{ NaClO}_4$ , saturated by argon in the presence of 3, 4 and 5% of PEGDA.

### 3.3 Possible crosslinking mechanism

Chemical composition of obtained hydrogels was confirmed by FTIR. Fig. 7 presents absorption spectra of CS (characteristic bands marked with dashed line), PEGDA (solid line) and dried hydrogels of these two components. The main characteristic absorption bands for chitosan are  $3455 \text{ cm}^{-1}$  (O-H stretching),  $2920$  and  $2875 \text{ cm}^{-1}$  (C-H stretching),  $1640 \text{ cm}^{-1}$  (N-H bending),  $1154 \text{ cm}^{-1}$  (anti-symmetric stretching of C-O-C bridge) and  $1094 \text{ cm}^{-1}$  (C-O stretching). PEGDA has characteristic bands at  $1740 \text{ cm}^{-1}$  (C=O stretching, in ester group),  $1092 - 1100 \text{ cm}^{-1}$  (C-O-C stretching) and weak band at  $810 \text{ cm}^{-1}$  (C=C stretching) [28]. Main characteristic peak of chitosan and PEGDA shared similar position in spectra of hydrogels. Despite their overlapping it was possible to distinguish chitosan in the hydrogels due to widening of peak about  $1000 \text{ cm}^{-1}$  (C-O stretching) presence of weak methylene band  $2920 \text{ cm}^{-1}$  (C-H stretching in  $\text{CH}_2\text{OH}$ ) and broad band from amide group ( $580 \text{ cm}^{-1}$  C=O out-of-planar bending) visible in the spectra [29,30].

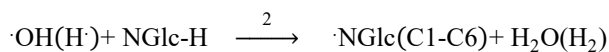
The composition of CMCS-based hydrogels was also evaluated by FTIR. Fig. 8 shows absorption spectra of untreated CMCS, hydrogels of PEGDA and of CMCS-PEGDA, where some characteristic bands of CMCS were marked with dashed lines and PEGDA with solid lines. Characteristic peaks derived from CMCS are located at  $1066 \text{ cm}^{-1}$  (C-OH stretching vibrations),  $3400 \text{ cm}^{-1}$  (stretching vibration of NH and OH),  $1603 \text{ cm}^{-1}$  - OH asymmetric stretching vibration (from the COOH group),  $1420 \text{ cm}^{-1}$  - symmetric stretching vibration of COOH,  $2900 \text{ cm}^{-1}$  and  $2875 \text{ cm}^{-1}$  (stretching vibration of CH,  $705 \text{ cm}^{-1}$  vibration  $\text{NH}_2$  group, and  $560 \text{ cm}^{-1}$  - stretching vibration of plane C = O amide group [31,32]. In the spectra of the obtained hydrogels, bands derived from CMCS partially overlap with signals from PEGDA, but there are visible bands from the CMCS at  $705 \text{ cm}^{-1}$  and  $599 \text{ cm}^{-1}$  that are not seen in the spectrum of PEGDA, despite irradiation dose applied. The other bands characteristic for CMCS at  $1630$ ,  $1420$  and  $1066 \text{ cm}^{-1}$  are also of wider shape than the overlapping signals from PEGDA. Last but not least, the band at  $2920 \text{ cm}^{-1}$  is also more pronounced than that of PEGDA. FTIR spectra of hydrogels demonstrated the presence of both components CMCS and PEGDA. This supports the results of sol-gel analysis, since such high content of insoluble part evidenced presence of the polysaccharide in the hydrogel.

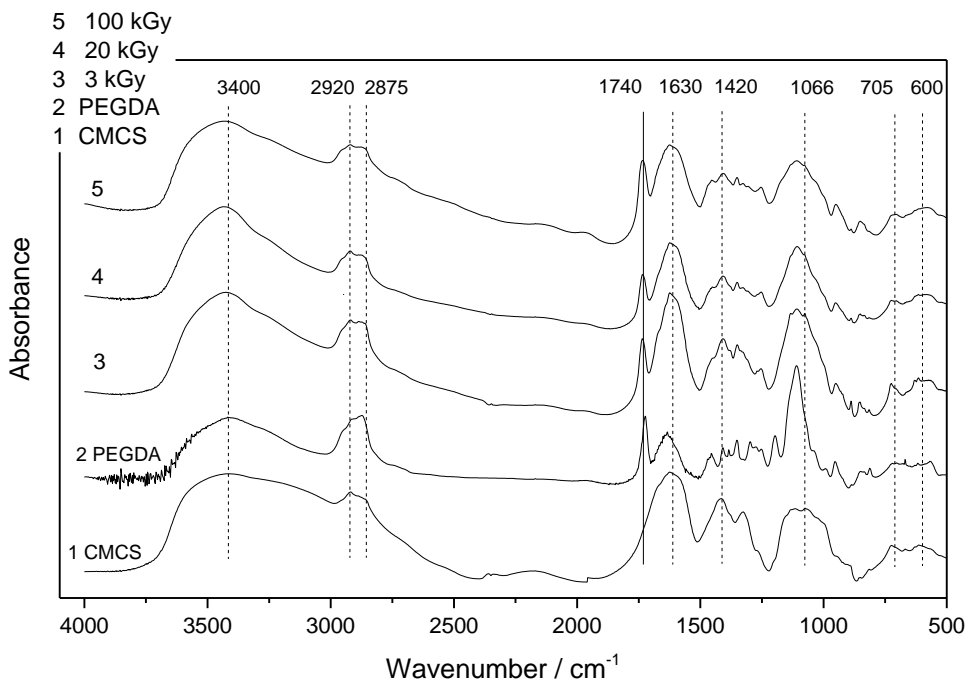


**Figure 7.** FTIR absorption spectra of chitosan, hydrogels of PEGDA, and hydrogels of chitosan – PEGDA obtained by irradiation of chitosan (concentrations indicated in the graph) and PEGDA (5%) in argon-saturated acidic aqueous solution (pH 3) with a dose of 10 kGy.

Considering state of the art of the radiation chemistry of polysaccharides and the results of present investigations we proposed a mechanism of crosslinking reactions. It is necessary to mention that in solutions containing chitosan (or CMCS) and PEGDA a Michael-type addition of  $\alpha,\beta$ -unsaturated carbonyl groups to amino group of chitosan catalyzed by acetic acid is probable [33,34]. This reaction may take place in water solution with high yield only at temperatures over 60°C. Thus, Michael-type addition reaction might occur only in limited extent, and did not lead to formation of a polysaccharide network. Despite of ageing time of the CS/CMCS – PEGDA solutions, entire samples were in the liquid state - sol.

As mentioned above radiation energy is mostly absorbed by water and direct effect of radiation on the polymer is minor. Then, the polysaccharide and PEGDA react with these transient products of water radiolysis, among which the most reactive towards typical carbohydrates is the hydroxyl radical  $\cdot\text{OH}$  ( $\cdot\text{H}$  acts in a similar way). It rapidly abstracts hydrogen atoms from C<sub>1</sub>-C<sub>6</sub> positions in glucosamine (NGlc) ring of a polysaccharide what leads to a macroradical, i.e., a radical located at the polysaccharide chain (reaction 2), which undergoes subsequent reactions [35].

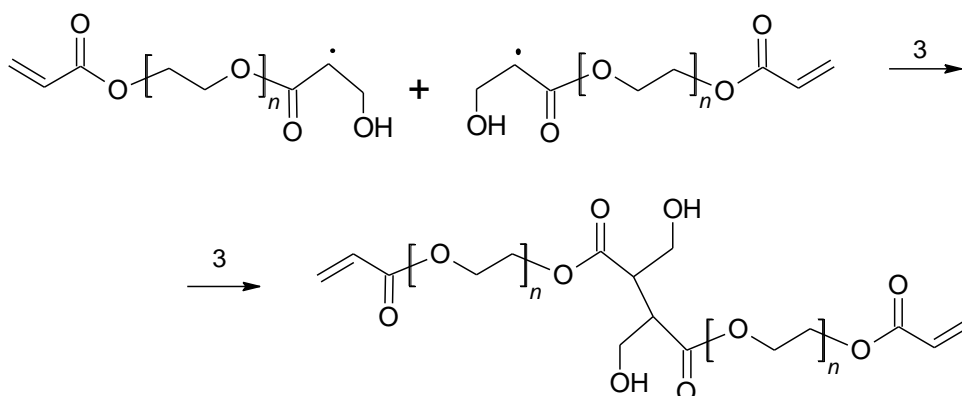


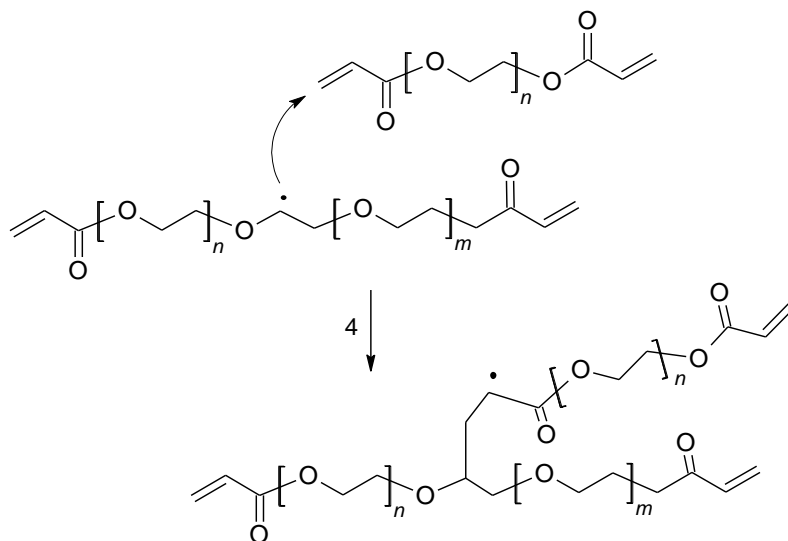


**Figure 8.** FTIR absorption spectra of original CMCS, hydrogels of PEGDA, and hydrogels formed by irradiation of 3, 20, 100 kGy of 5% CMCS with 3% PEGDA in 0.1 M NaClO<sub>4</sub> aqueous solution.

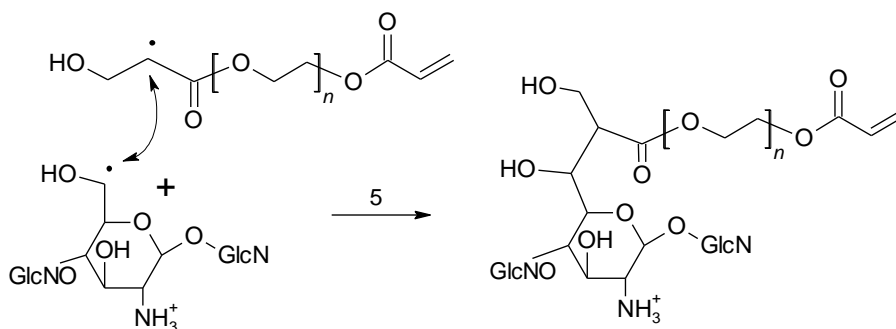
Radicals created on PEGDA participate in polymerization and simultaneous crosslinking by addition of the radical located on the main chain or terminal acrylic groups to unsaturated double bonds (reactions 3 and 4) or the main chain of the another macromonomer molecule [25].

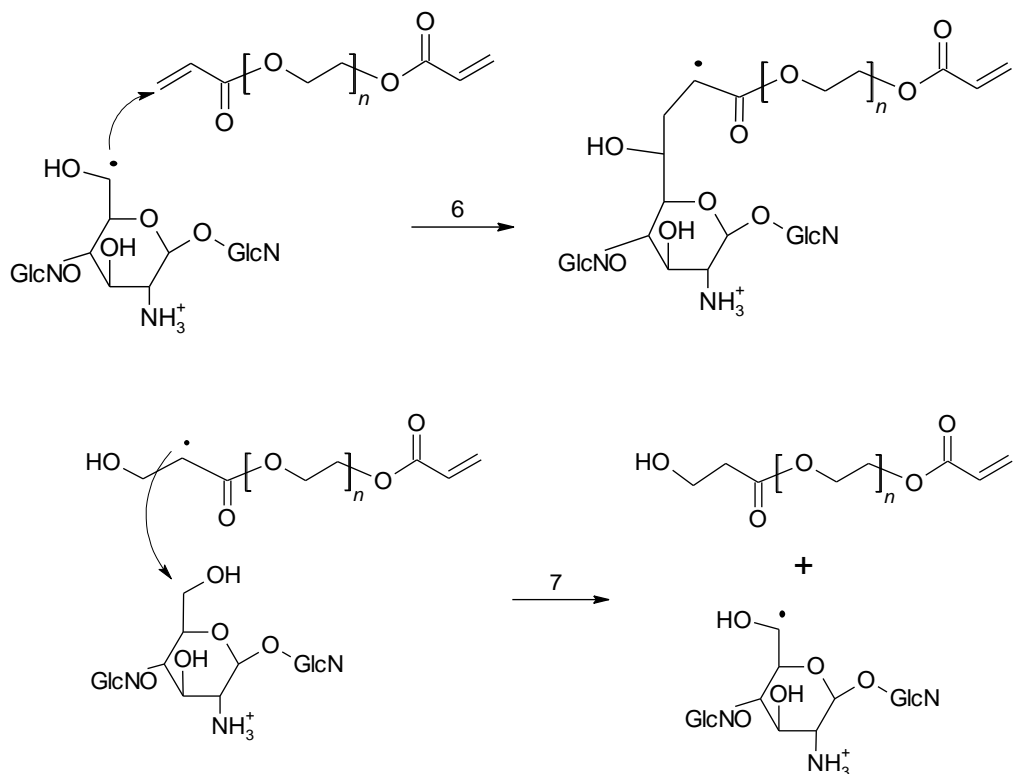
Reaction between PEGDA radicals occurs probably more frequently than chitosan radical addition due to the instability of chitosan radicals and their tendency to disproportionate with double bond formation [19]. Even if crosslinking reactions involving chitosan are less efficient, the polysaccharide remains in the hydrogel matrix mainly because the addition of one glucosamine unit to the network results in bounding of the whole chitosan chain.



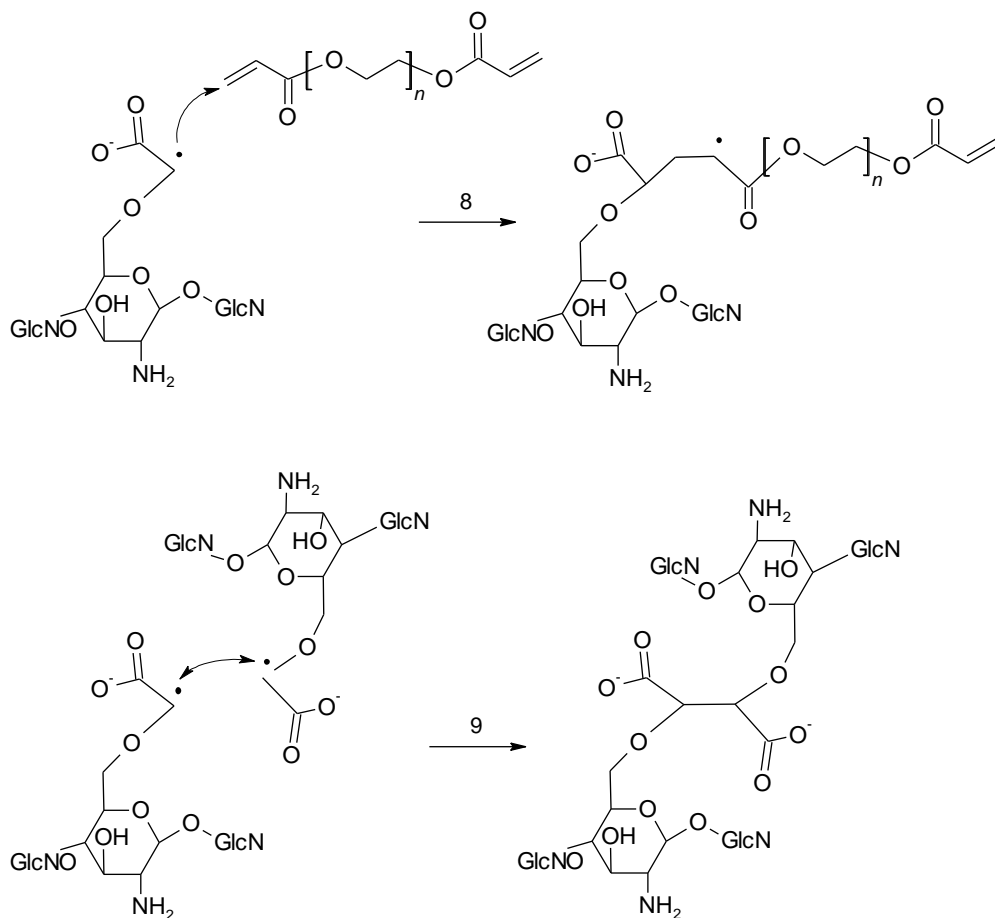


Radical can be located in every position on glucosamine monomer unit but with somewhat different probability because C<sub>2</sub> and C<sub>3</sub> position are protected against hydroxyl radical attack by neighboring protonated amino group. Formation of macroradicals on C<sub>1</sub> and C<sub>4</sub> position results mainly in main chain scission (by breaking the 1-4 glycosidic bond between glucosamine units) so it was deduced that the most probable mechanism of chitosan crosslinking reaction with PEGDA involves C<sub>6</sub> atoms. These can be recombination of a CS macroradical with PEGDA radical (reaction 5, for simplicity all schemes shows PEGDA radicals located only on terminal beta carbon with respect to hydroxyl group) or the attack of CS macroradical to PEGDA double bond (reaction 6). Radical transfer from PEGDA to CS chain is also probable (reaction 7) but this does not directly influence degradation nor crosslinking of the polysaccharide.



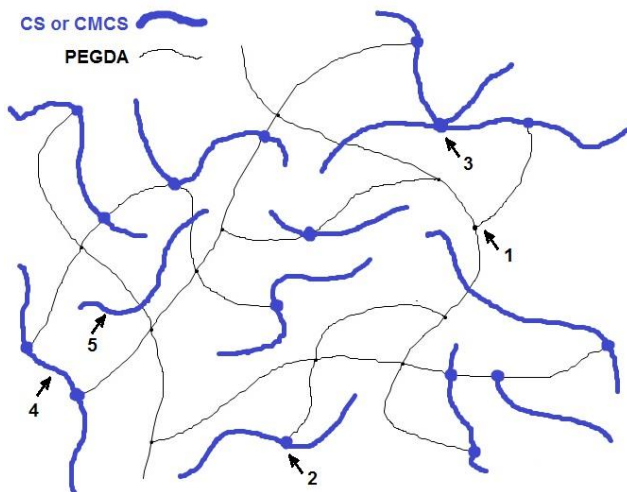


There are two the most probable pathways of the crosslinking involving CMCS and PEGDA. Similarly to CS described beforehand, radicals positioned at C<sub>6</sub> carbon atom of the glucopyranose monomer unit can be involved in attacking double bond of PEGDA or recombine with PEGDA radicals. Alternative mechanism engages radicals on the α-carbon atom with respect carboxyl group in the side chain that can react with the acrylic group of PEGDA (reaction 8) or recombine with PEGDA radical. These reactions may be even more probable due to high degree of substitution of the chitosan derivative of 96%, i.e. every monomer unit in average bears 1.92 carboxymethyl moieties, therefore nearly twice than the C<sub>6</sub> atoms. Moreover carboxymethyl side chains are somewhat projected out of the backbone which facilitates access to other molecules or less steric hindrance. Besides, classical CMCS crosslinking through recombination of radicals located at α-carbon atoms of carboxymethyl groups occurs as well (reaction 9), and increases the yield of gel formation by the crosslinker – ca. 80% of gel fraction (Fig. 6a).



The reactions presented above do not expend possibilities of radiation induced reactions in the investigated systems because, among others, chitosan or CMCS radicals centered in other carbon atoms, even those resulted from main-chain scission, have also ability to attack PEGDA double bonds.

Possible architecture of the hydrogel presented in Fig. 9 may comprise a network of crosslinked PEGDA (1), with polysaccharide chains bounded mainly to the end groups of macromonomer (2). Since polysaccharides undergo main chain scission, participation of the polysaccharide chain in formation of the network is less probable, (4). Regular crosslinking points of CMCS (through alpha carbon atoms of carboxymethyl groups) occur as well (3). The network may also embed, not chemically crosslinked, polysaccharide macromolecules (5).



**Figure 9.** Possible network architecture of polysaccharide chains crosslinked with PEGDA. Numbers represent crosslinking points of (1) PEGDA-PEGDA; (2) & (4) PEGDA-polysaccharide; (3) CMCS-CMCS; and (5) free or entangled polysaccharide chain.

#### 4. Conclusion

Results of this study indicate that ionizing radiation is a convenient tool to synthesize hydrogels based on chitosan and its derivative of carboxymethyl chitosan. By selecting experimental conditions, such as concentration of a polysaccharide and the crosslinking agent, it is possible to change dominant pathway of radiation-induced reactions from degradation into crosslinking. Especially interesting is that the method presented here allows formation of macroscopic gels even from a polysaccharide solution of low concentration, which is distinct from the method of radiation-induced polysaccharide crosslinking in the paste-like state, known from earlier literature – in the latter technique only in highly concentrated (few tens %) solutions of water-soluble polysaccharides crosslinking predominated over degradation.

Addition of 5% PEGDA to chitosan and CMCS solutions resulted in maximum obtained gel fractions of ca. 80%. Moreover, by application of a certain, predetermined dose to the selected formulation one can obtain hydrogels with desired water uptake capacity, which may be in the range of 20 to 500 g of water hold by 1 gram of gel. What is more, if applied dose are of 25 kGy or more, the gels may be manufactured and sterilized simultaneously during the synthesis, combining gel formation and sterilization into a single process.

FTIR spectra of dried hydrogels of chitosan (carboxymethyl chitosan) – poly(ethylene glycol) diacrylate confirmed the presence of the polysaccharide in the structure of hydrogels. The possible crosslinking mechanisms were proposed, involving radicals on the polysaccharide C<sub>6</sub> carbon atoms, or in the case of CMCS also on alpha carbon atom of the carboxymethyl substituent, and the double bonds of PEGDA. Even though the polysaccharide chains may be partly degraded during irradiation, it may be beneficial for potential application of the hydrogel as wound dressing since chitosan or its derivative of relatively low molecular weight demonstrate antimicrobial activity.

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