SYNTHESIS AND POTENTIAL CYTOTOXICITY EVALUATION OF CARBOXYMETHYL CHITOSAN HYDROGELS

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Abstract

The aim of the research was to employ radiation to produce flexible carboxymethyl chitosan (CMCS) based hydrogels of uniform structure to characterise their swelling properties and cytocompatibility for potential applications as hydrogel wound dressings. CMCS in aqueous solution was irradiated with an electron beam in the presence of a poly(ethylene glycol) diacrylate (PEGDA) macromonomer as a crosslinker, at 12 different compositions, i.e. 3–20% CMCS, 3 and 5% PEGDA. The obtained hydrogels were subjected to sol–gel analysis. The amount of insoluble fraction (up to 100%) rose with an increase in the PEGDA/polysaccharide ratio. Moreover, the equilibrium degree of swelling, ca. 15 to 200 g of water per g of gel, which was higher for lower content of crosslinker, decreased with the delivered dose, which was associated with an increase in crosslinking density. The in vitro XTT cell viability assay (murine fibroblasts, L929 cell line) showed no significant cytotoxicity of CMCS gels.

Key words: hydrogel, carboxymethyl chitosan, radiation crosslinking, PEGDA, XTT, cytotoxicity

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1. Introduction

Hydrogels are materials characterised by solid matter properties: they typically possess a specific shape and have measurable mechanical properties, as well as liquid-like features, which allows for easy absorption, penetration and release of molecules. Hydrogels are used in medicine, such as in systems for the controlled release of drugs, wound dressings, surfaces and matrices for tissue culture and tissue engineering.

Hydrogel dressings are applied to burns, pressure sores, ulcers, surgical wounds and other types of skin lesions. They isolate the wound from the external environment, constituting a barrier from 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 with three-dimensional structures of mutually linked polymer chains filled with water or water-based solvents. A leading industrially implemented technology, allowing the formation and sterilisation of hydrogel dressings in a single step, is based on the use of ionising radiation. Applying radiation to aqueous solutions of selected polymers allows for simultaneous crosslinking (hydrogel formation) and, if a sufficiently high dose is applied, sterilisation. So far, this method is mainly used in manufacturing hydrogel dressings based on biocompatible hydrophilic synthetic polymers [1].

The use of natural polymers as the main component of the hydrogel has many advantages associated with their biocompatibility and biological properties, but their disadvantages include not always having perfect chemical purity, batch to batch reproducibility of the substrate and the formation of supramolecular structures with limited solubility. The use of hydrophilic substituents to increase the solubility of certain polysaccharides facilitates crosslinking of chains by various methods, including ionising radiation [2–5].

Chitosan (CS) (Fig. 1) is an amino polysaccharide: a deacetylated product of chitin. Chitin is a renewable and biodegradable carbohydrate polymer obtained from the exoskeletons of shellfish or insects. CS is composed of N-acetyl-D-glucosamine and D-glucosamine units connected by β-(1-4) bonds [6–8]. It possesses various physicochemical and biological properties, and is therefore widely used and investigated for applications in biomedicine, pharmaceuticals or cosmetics, for example as a wound healing promoter or drug carrier [6, 9]. However, CS is soluble only in acidic solutions (pH below 6.5), it presents an uncontrollable rate of degradation and its hydrogels do not show high water binding capacities [6, 7, 10–12]. Due to its restricted solubility and degradation, many derivatives, such as carboxymethyl chitosan, chitosan esters, N-trimethylene chloride chitosan, have been synthesised and investigated [10]. One of the best chitosan derivatives is carboxymethyl chitosan (CMCS) (Fig. 2), which has several advantages over its parent chitosan. CMCS possesses an ampholytic character, due to the presence of amino and carboxyl moieties. Depending on the environment, CMCS may present different ionisation states that improve its solubility and therefore widens its potential use in medicine [6, 7]. Moreover, it exhibits low toxicity, biodegradability, excellent biocompatibility, improved antioxidant property, stability in blood and has the ability to form hydrogels [9, 11, 13, 14]. CMCS is used in numerous materials as moisture-retention agents, bactericides, wound dressings, artificial tissue, blood anticoagulants or drug-delivery matrices [9, 15]. It is postulated that CMCS can stimulate the extracellular lysozyme activity of fibroblasts, proliferation promotion of normal skin fibroblasts and inhibit the proliferation of keloid fibroblasts [9].
In the present research, poly(ethylene glycol) diacrylate (PEGDA) was used as a crosslinking agent to form CMCS hydrogels by a radiation method. The possible reaction pathways involving PEGDA and CMCS were proposed previously by Czechowska-Biskup et al. [7]. We assumed that CMCS hydrogels would present a low cytotoxic response since chitosan is a non-toxic polymer. In order to confirm this assumption, an XTT [16, 17] assay was employed to assess potential toxicity of the gels on mouse fibroblasts (L929 cells). The manufactured hydrogels are anticipated to be applied as wound dressings. The currently obtained in vitro biocompatibility results will be crucial to perform further in vivo tests.

2. Materials and Methods

2.1. Chemicals/materials

Carboxymethyl chitosan (CMCS) of the degree of deacetylation (DA) and the degree of substitution 93.8% and 91.2%, respectively, was purchased from Kraeber & Co GmbH, Germany. Poly(ethylene glycol) diacrylate (PEGDA), MW = 700 g·mol⁻¹, and high purity grade sodium perchlorate monohydrate (NaClO₄·H₂O), were purchased from Sigma-Aldrich. High-purity water (0.055 µS cm⁻¹, TKA MicroPure system) was used in all experiments.

2.2. Preparation of solutions and gel manufacturing

Solutions of CMCS were prepared by dissolution of the appropriate amount of CMCS in 0.1 mol·dm⁻³ solution of NaClO₄ and stirring at room temperature for 72 h. 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, 3 or 5% PEGDA was added. Plastic bags were filled with approximately 1 g of polymer solution and heat sealed. Samples were irradiated at room temperature with 6 MeV electron beam (EB) from a linear accelerator (Elektronika ELU-6e, Russia). The average dose rate was ca. 5 kGy·min⁻¹ as determined by alanine dosimetry. Doses in the range from 1 to 100 kGy were applied.

2.3. Sol–gel analysis

The formed hydrogels were placed in excess of water, which was changed daily in order to extract the soluble fraction (sol). After a week, swollen hydrogels were weighed for determination of the equilibrium degree of swelling (EDS) calculated using equation 1. Subsequently, hydrogels were dried in an oven at 40°C, initially at atmospheric conditions.
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pressure and afterwards under vacuum. Finally, they were weighed and the gel fraction (GF) was calculated with equation 2.

\[ EDS = \frac{W_s - W_d}{W_d} \]  
\[ GF = \frac{W_d}{W_0} \cdot 100\% \]

where:

- \( W_s \) 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 the ‘sol’ part (i.e. the weight of a dry polymer network),
- \( W_0 \) is the dry mass of initially used substrates, i.e. the polysaccharide and PEGDA.

2.4. Cell culture

The mouse fibroblast cell line L929 (Sigma Aldrich) was chosen to evaluate the biocompatibility of CMCS hydrogels. Cells were grown in Minimum Essential Medium (MEM) (Biowest) containing 10% foetal bovine serum (Sigma Aldrich), 1 mM sodium pyruvate (Sigma Aldrich), 0.5 mM non-essential amino acids (NEAA) (Sigma Aldrich) and 100 units/mL penicillin and 100 mg/mL streptomycin (Sigma Aldrich) in an incubator at 37°C with 5% CO₂. Cells were plated and grown to 80% confluency before the initiation of the assay.

2.5. XTT cytotoxicity assay

The XTT assay was used to evaluate cell metabolic activity. In viable cells, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) is metabolically reduced to a water-soluble orange formazan product. The number of viable cells is correlated with the colour intensity determined by photometric measurements. XTT (Serva) was dissolved in MEM without phenol red (Gibco) at a concentration of 1 mg/mL. Phenazine metosulphate (PMS) (Serva) was dissolved in PBS buffer to reach a concentration of 5 mM. PMS solution was then added to the XTT solution at a concentration of 25 µM.

Samples for testing were prepared in accordance with ISO 10993-12 “Biological evaluation of medical devices – Sample preparation and reference material” and cytotoxicity was evaluated with respect to ISO 10993-5 “Biological evaluation of medical devices—Part 5: Tests for in vitro cytotoxicity”. The indirect method was applied – the test was performed on extracts of the samples. The chosen hydrogels were incubated with the extraction medium (culture medium with serum) at a concentration of 0.1 g/mL, plus an extra amount of medium that 0.1 g material absorbs during the test, for 24 h at 37°C in a shaking incubator. After that time, the cell culture medium was removed and replaced by sample extract medium. Then the cells were incubated at 37°C with 5% CO₂ for 24 h prior to the XTT component addition. As a negative control, 10x diluted Triton X-100 (Sigma-Aldrich) was used. For a positive control, which does not induce a cytotoxicity effect, low-density polyethylene (Sigma-Aldrich) was used. Polyethylene was incubated with the culture medium with serum for 24 h at 37°C at a concentration of 0.2 g/mL. Cells cultured under normal conditions, without any other material were assumed as the blank control.
For the assay, cells were seeded into 96-well plates at a density of $10^4$ cells per well and cultured for 24 h at 37°C and 5% CO$_2$. Then the medium was removed and cells were exposed to 100 µL of positive and negative controls and to 100 µL of the material extract over a range of dilutions or nothing but culture medium (blank control). After 24 h of cell incubation with the material extracts, 50 µL of the XTT/PMS solution was added to each well and incubated in the dark for an additional 3 h at 37°C. Then the plates were swayed and an aliquot of 100 µL from each well was transferred into the corresponding well of a new plate. Next, the absorbance was measured by using a microplate reader (Synergy H1, BioTek, Winooski, VT, USA) at a wavelength of 450 nm. The cell viability was expressed as a percentage of the control values (blank). The experiments were performed in triplicate.

3. Results and Discussion
3.1. Formation and analysis of the gels

Chitosan and its derivatives can be crosslinked to form a network by a chemical method using crosslinking agents, for instance glutaraldehyde, genipin or tripolyphosphate [7]. The chemical method of hydrogel synthesis is highly complex, as it requires procedures involving the presence of a catalyst, thermal initiation and purification of the product. Therefore, the use of radiation to initiate crosslinking and form a gel, either with or without a crosslinking agent is less complicated and more efficient. It is also faster, lasts for only seconds or minutes depending on the required dose and dose rate. Formation of hydrogels based on polysaccharides is feasible using ionising radiation. Nevertheless, to achieve efficient crosslinking of CMCS in aqueous solutions of concentrations below 10%, the addition of a crosslinking agent is necessary. Therefore, we selected a macromonomer of poly(ethylene glycol) diacrylate (Fig. 3) as a crosslinker in this study, because it is effective in interchain linkage formation through its terminal double bonds, and, importantly for intended biomedical applications, it has low toxicity. Furthermore, PEGDA polymerises and forms a network when irradiated with relatively low doses in aqueous solution [18–22].

![Structure of poly(ethylene glycol) diacrylate](image)

Based on the literature, the concentrations of PEGDA of 3 and 5% were chosen for experiments with carboxymethyl chitosan, and irradiation at a dose range of 1 kGy to 100 kGy was applied [7]. CMCS processed at even the relatively low concentration (Table 1) produced hydrogels at the presence of 3 and 5% PEGDA. The sol–gel analysis of the obtained hydrogels indicates the effect of CMCS concentration in irradiated solutions. The gel fraction and EDS of the hydrogels are presented in Fig. 4–5 and 6–7, respectively.
Table 1. Carboxymethyl chitosan (CMCS) and poly(ethylene glycol) diacrylate (PEGDA) concentrations used in the study

<table>
<thead>
<tr>
<th>PEGDA concentration [%]</th>
<th>CMCS concentration [%]</th>
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<tr>
<td>3</td>
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<td>5</td>
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<td>15</td>
<td>15</td>
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<td>20</td>
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The content of gel in the network formed with 3% PEGDA was significantly higher at lower CMCS concentrations, and this was particularly distinctive at lower irradiation doses. However, the GF remained nearly unchanged for lower CMCS concentrations (<10%), but it increased gradually with radiation dose in higher CMCS concentration systems (>10%). At higher CMCS concentrations, the crosslinking agent was not able to link most polysaccharide chains, especially at lower doses. The energy absorbed by the system is divided into the solutes according to their concentrations, or strictly their ratio, and reaction rate constants. The main initiator of radicals on the CMCS and PEGDA macromonomer resulted from water radiolysis was the hydroxyl radical. The OH radical rate constant with PEGDA is much higher than that with CMCS [18]. The fact that the CMCS forms hydrogels at high concentrations only at high doses, above 20–30 kGy, is also taken under consideration. Therefore, a higher radiation dose (above 25 kGy) was necessary to form a chemical gel from higher CMCS concentration solutions even in the presence of PEGDA.

Figure 4. Gel fractions of hydrogels formed from carboxymethyl chitosan at various concentrations and 3% poly(ethylene glycol) diacrylate as a function of the irradiation dose

The trend of insoluble fraction content for hydrogels formed with 5% PEGDA solutions was similar for all CMCS concentrations and doses to that for 3% PEGDA: it
decreased with increased CMCS concentrations. At low radiation doses, the amount of gel fraction was several times higher than that at 3% of the crosslinking compound. This is due to the neat CMCS effectively not crosslinking at low doses (it does but to a lesser extent than to form a macroscopic gel). Therefore, the addition of PEGDA allows a gel to be obtained even at lower radiation doses. The higher ratio of PEGDA to CMCS results in an increased gel fraction, as compared to the 3% PEGDA system. Previous studies have shown that the entire PEGDA, when irradiated in aqueous solutions at low percent concentration, contributes to the gel fraction, and therefore a small addition of CMCS of 3 and 5% does not change this significantly [7,22].

Hydrogels swelled by absorbing a large amount of water. The EDS may be controlled in the range of 15 to 200 (g of water per g of gel) through selection of ingredient composition and by absorbed dose, which determined crosslinking density. Fig. 6 shows that the amount of water absorbed by gels formed from 3% PEGDA and CMCS solutions depended on the polysaccharide concentration and the irradiation dose. A minor influence of the dose was detected for the lowest concentrations of CMCS, 3 and 5%, which swells by absorbing ca. 40 and 50 g of water per g of gel, whereas a diverse effect of dose was observed for gels of higher CMCS concentrations. The gels containing 10% CMCS revealed lower water uptake, at doses below 15–20 kGy, than the EDS increased. Since PEGDA underwent quick crosslinking also involving CMCS participating in the network, the swelling was lower. However, with further irradiation, the relatively inelastic network softens by partial degradation of the polysaccharide, which in turn controls swelling. At 15% of CMCS, classical crosslinking behaviour is observed, demonstrated by an increase in the GF and a reduction of the EDS with irradiation dose as the density of the network gradually increases. Solutions of 20% CMCS crosslinks in similar manner (compared to GF), but a very high content of the polysaccharide controls the swelling, and this is responsible for the maximum observed in the moderate doses.

![Graph showing gel fractions of hydrogels formed from carboxymethyl chitosan at various concentrations and 5% poly(ethylene glycol) diacrylate as a function of the irradiation dose](image-url)

**Figure 5.** Gel fractions of hydrogels formed from carboxymethyl chitosan at various concentrations and 5% poly(ethylene glycol) diacrylate as a function of the irradiation dose
The EDS presented in Fig. 7 shows that the radiation dose had no significant effect on the EDS value of the hydrogels formed from solutions containing 5% PEGDA. In general, the values of swelling lower than in the case of 5% PEGDA compositions, e.g. below 30 (g/g), were controlled by the high concentration of the crosslinker macromonomer, yet the EDS was inversely proportional to the CMCS concentration. The average EDS value for such solutions is 15 to 25 g of water per 1 g of dry gel from the highest to the lowest content of the polysaccharide. This indicates the network density was lower for higher CMCS/crosslinker ratios.

Therefore, the radiation treatment gave the possibility to obtain the desired water uptake of the gels. It can be easily adjusted to individual needs through the choice of output mixture composition and the application of a specific radiation dose.

**Figure 6.** Equilibrium degree of swelling (EDS) of hydrogels formed from carboxymethyl chitosan at various concentrations and 3% poly(ethylene glycol) diacrylate as a function of the irradiation dose

**Figure 7.** Equilibrium degree of swelling (EDS) of hydrogels formed from carboxymethyl chitosan at various concentrations and 5% poly(ethylene glycol) diacrylate as a function of the irradiation dose
3.2. Cyto compatibility assessment

XTT test is a sensitive *in vitro* assay to measure cell viability being in contact with a tested biomaterial. Mouse fibroblast L929 cells were incubated with extracts of hydrogels formed through irradiation of 3 or 5% PEGDA, with 5, 10, 15, 20% of CMCS with 25 kGy. The system comprising CMCS and PEGDA may induce a slightly toxic effect if a higher amount of crosslinker is used (Table 2). In the case of 5% PEGDA compositions, for higher CMCS concentrations, the fewer viable cells were determined: the cell viability reached 68.8% for the 20% CMCS hydrogel during incubation with extracts. The same correlation was observed for 3% PEGDA hydrogel compositions, though if cell viability is over 70% (in this case 84.3%), the material is regarded as non-toxic. In reference to the aforementioned standard ISO 10993-5, at least four different concentrations of the hydrogel extracts were prepared and evaluated. It was especially important to determine the 50% extract of the test sample to check if it had at least the same or a higher viability than the undiluted extract in order to confirm the correctness of the method.

Results of cell viability of cells in contact with extracts of CMCS hydrogels are presented in Fig. 8–13. Fig. 8 and Fig. 11 show the correlation between CMCS concentrations and cell viability for 5 and 3% PEGDA hydrogels, respectively. The details of cell viability with respect to the blank, negative and positive controls are presented in Fig. 9 (3% CMCS/5% PEGDA), 10 (20% CMCS/5% PEGDA), 12 (3% CMCS/3% PEGDA) and 13 (20% CMCS/3% PEGDA).

<table>
<thead>
<tr>
<th>CMCS concentration [%]</th>
<th>Viability PEGDA 3%</th>
<th>Standard deviation</th>
<th>Viability PEGDA 5%</th>
<th>Standard deviation</th>
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<tr>
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<td>7.6</td>
<td>58.3</td>
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<td>84.3</td>
<td>8.4</td>
<td>68.8</td>
<td>1.4</td>
</tr>
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</table>
Figure 8. Cell viability at different carboxymethyl chitosan (CMCS) concentrations and 5% poly(ethylene glycol) diacrylate.

Figure 9. Cell viability for 3% carboxymethyl chitosan and 5% poly(ethylene glycol) diacrylate hydrogel.
Figure 10. Cell viability for 20% carboxymethyl chitosan and 5% poly(ethylene glycol) diacrylate hydrogel

Figure 11. Cell viability at different carboxymethyl chitosan (CMCS) concentrations and 3% poly(ethylene glycol) diacrylate
Figure 12. Cell viability for 3% carboxymethyl chitosan and 3% poly(ethylene glycol) diacrylate hydrogel

Figure 13. Cell viability for 20% carboxymethyl chitosan and 3% poly(ethylene glycol) diacrylate hydrogel
Cells incubated with 1% Triton X-100 (negative control) showed an extremely large decrease in viability. The positive control presented viability of around 80–90%. In all cases, cell viability was independent of the CMCS concentration (however, a minor trend for its decrease with an increase in polysaccharide content was perceptible), as measured for undiluted hydrogel extracts. Dilution of the extract resulted in a somewhat increased viability, even above 100% after one day of incubation. Moreover, inversely to the 100% extract, its dilutions did not present lower viability while increasing the CMCS concentrations.

The presence of the crosslinking component is critical for the safety of the gels. Hydrogels of the 3% PEGDA revealed higher viability – over 80%, than those containing 5% of the crosslinking agent. At the highest CMCS concentration, 5% of PEGDA showed a significant decrease in cell viability, whereas at the lowest polysaccharide content, the cell viability was also reduced but to a lesser extent. Clearly, the effect of the remaining double bonds of the crosslinking agent is exposed here. As mentioned earlier, a weight ratio of the two components being irradiated in the solution determine the ratio of energy absorbed by each of them. At low CMCS concentrations, the majority of energy is consumed for the reactions of PEGDA; therefore, potentially toxic unsaturated terminal bonds are chiefly utilised. Conversely, PEGDA participates in energy absorption to a lesser extent at high concentrations of the other solute, which resulted in a large number of unreacted double bonds and thus a higher toxicity of the compositions with greater CMCS/PEGDA ratios.

In summary, the results showed no cytotoxicity effect of the polysaccharide, but cytotoxic effects were found for the crosslinking agent. This will define the design of new materials for further studies. Considering in vivo experiments using small rodents to evaluate material interaction with living tissue, hydrogels manufactured with the lowest possible crosslinking agent content should be used.

4. Conclusion

The results presented in this report demonstrate that ionising radiation is a suitable tool to synthesise hydrogels based on carboxymethyl chitosan. By selecting experimental conditions, such as the concentration of the polysaccharide and the crosslinking agent, it is possible to change the dominant reaction pathway from degradation, which takes place while CMCS is irradiated in the solid state and in dilute solutions, into crosslinking. The addition of PEGDA to CMCS solutions processed by radiation resulted in the formation of firm macroscopic gels, even at relatively low irradiation doses of a few kGy, and the GF increased with the increase in the PEGDA/CMCS ratio. Of great importance, through the application of certain initial formulations, i.e. composition of water-soluble ingredients, and the specific dose, the hydrogels with the desired water absorption capacity (in the range from approx. 15 to approx. 200 g retention of water per 1 g of gel) can be obtained. Furthermore, when the applied dose is 25 kGy or higher, the gels may be formed and sterilised simultaneously during the production step, taking advantage of radiation processing. Gel formation is therefore synthesised and sterilised in a single technological process.

Since the anticipated application of manufactured hydrogels is a biomedical field, a crucial factor is their safety for the organism. Therefore, as a first step, biocompatibility and viability of cells in contact with gel extracts was evaluated. Hydrogels did not show significant cytotoxicity, and even an increase in the number of cells was observed at lower extract concentrations. Some toxic effects were revealed due to the presence of the crosslinking agent, e.g. the hydrogels containing 5% PEGDA were less cytocompatible.
in comparison with those comprising 3% PEGDA. Therefore, a low amount of PEGDA in the hydrogel is fundamental for the biological safety of the biomaterial. This is specifically related to the number of unreacted double bonds in PEGDA terminals. Thus, increasing the concentration of PEGDA, or the polysaccharide to macromonomer ratio will result, at a specific irradiation dose, in a greater number of retained double bonds. When designing a medical hydrogel product, one should select a composition with lower concentration of macromonomer and apply a relatively high irradiation dose in order to exhaust virtually all reactive double bonds of PEGDA, preferably in crosslinking reactions. Due to the addition of synthetic components of PEGDA, the hydrogels are not fully biodegradable. Therefore, the produced construct may be considered in wound care applications as a dressing. In vivo tests on small animals will be carried out in order to further assess the biocompatibility of gels comprised of CMCS.

5. Acknowledgements

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6. References


