Controlled modification of hyaluronic acid for photoinduced reactions in tissue engineering.

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Abstract
Implementation of up-to-date 3D printing methods for tissue engineering applications requires synthesis of novel materials possessing both biocompatibility and processability properties. Since the hyaluronic acid is an endogenous material it represents an attractive candidate for scaffold formation. In this work, chemical modification of hyaluronic acid with glycidyl methacrylate (HAGM) was used to produce derivatives that are capable of photoinduced cross-linking. Aiming to control the degree of substitution in hyaluronic acid by glycidyl methacrylate we developed a new quantitative method based on oxidation of double bonds in HAGM by permanganate ions. HAGM hydrogel scaffolds have been produced and examined as in vitro and in vivo.

1. Introduction
Tissue engineering is a branch of regenerative medicine serving to create artificial implants that can functionally replace damaged organs and tissues instead of using donor organs. Creation of an implant requires fabrication of 3-dimensional (3D) scaffolds that serve as a structural support for patient cells. Despite the rapid development of this approach, a comprehensive material for scaffolds that could meet all the requirements of native tissue has not been developed yet.

Materials (commonly polymers) for scaffold fabrication can be classified as natural, semisynthetic (derivatives of natural polymers) and synthetic. Alongside with wide variety and good reproducibility the most essential advantage of synthetic polymers is vast plasticity of physical chemical properties (e.g. poly(lactic acid)). Despite such benefits, most of synthetic polymers are often completely unsuitable for tissue engineering due to lack of biocompatibility and biodegradability that natural polymers have (e.g., collagen) [1]. Therefore, in order to combine the desired properties of both synthetic and natural polymers the development of new semisynthetic materials is needed.

Hyaluronic acid (HA), polysaccharide of extracellular matrix, appears to be promising material for scaffolding due to its endogenous origin [2]. However, it is impossible to produce hydrogel scaffolds out of non-modified HA via photocrosslinking. In this work we demonstrate the synthesis of HA derivatives, carrying double bonds, capable to crosslinking by flavin mononucleotide as a photosensitizer. The new rapid quantitative method has been implemented to control degree of
substitution (DS) in HA after modification. The synthesized hyaluronic acid glycidyl methacrylate (HAGM) has been used to produce hydrogel structures via photocrosslinking under irradiation at 365 nm wavelength. \textit{In vitro} and \textit{in vivo} tests of the produced hydrogels confirmed their biocompatibility.

2. Materials and methods

2.1. Materials

Following materials were purchased from Sigma-Aldrich (USA) and used without further purification: sodium hyaluronate with average molecular mass 1 MDa and 100 kDa, glycidyl methacrylate (GMA), triethylamine (TEA), tetraethylammonium bromide (TEAB), aceton, dimethylformamide (DMF), phosphate buffered saline, pH 7.0 (PBS), potassium permanganate (KMnO\textsubscript{4}), poly(ethylene glycol) diacrylate (PEG-DA) with average molecular weight 575 kDa, flavin mononucleotide (FMN).

2.2. Methods

2.2.1. Modification of HA

First, 0.5 g of HA was dissolved in 100 ml of PBS under vigorous stirring. Then, DMF was added with volume ratio PBS:DMF = 100:33. GMA was added in the range of concentrations to achieve mass ratio (HA:GMA) 1:6, 1:10, 1:15 and vigorously stirred. Then, 3 ml of TEA (catalyst) was added resulting to pH increase up to 12. Reaction was carried out for 7 days under stirring at room temperature. In modified method TEA was replaced with TEAB. Before introduction into the reaction mixture, 500 or 1000 mg of TEAB were dissolved in 5 ml of water and added to the mixture before GMA.

2.2.2. Detection of double bonds by KMnO\textsubscript{4} in GMA and HAGM

GMA was dissolved in deionized water at concentration 0.5 wt.%. After addition of GMA solution in the range of volumes from 10 to 70 μl to 0.025 wt.% KMnO\textsubscript{4} (standard) solution, the absorption spectra were recorded. The increase of added GMA volume was accompanied with color changing from violet to brownish yellow. Quantity of GMA and, correspondingly, double bonds needed to fully reduce permanganate ions was counted. This data will be used further to evaluate the degree of substitution (DS) of HAGM.

HAGM was dissolved in deionized water at concentration of 0.5 wt.% and then added to standard water solution of KMnO\textsubscript{4} (0.025 wt.%) until purple color completely turned into brownish yellow. To confirm complete disappearance of permanganate ions the solution was spectrophotometrically analyzed. DS of HAGM was evaluated as ratio of double bonds quantity (acquired previously from quantity of GMA needed to fully reduce 1 ml of standard KMnO\textsubscript{4} solution) to HAGM disaccharide quantity of samples.

All the absorption spectra were recorded at UV-Vis Spectrophotometer Evolution 201 (Thermo Fisher Scientific, USA).

2.2.3. Gelation of hydrogels

Photocurable compositions (PCCs) were produced by combining HAGM and PEG-DA in PBS and FMN as a photosensitizer. The final concentrations in the PCCs were as follows: 20 wt.% of HAGM, 5 wt.% of PEG-DA and 0.0004 wt.% of FMN. All the compositions were sonicated for 3 hours and left overnight under dark conditions up to the formation of uniform consistence.

Gelation of hydrogels due to activation of the crosslinking process in the produced PCCs was performed by UV diode “Vega” (Medlaz-Neva, Russia) emitting at 365 nm wavelength corresponding to the absorption peak of FMN. The intensity of irradiation was 20 mW/cm\textsuperscript{2}. 3D printing of hydrogel scaffolds was conducted according to the method described in [3], but irradiation was at 365 nm wavelength.
2.2.4. Cell culture

Immortalized human fibroblasts BJ-5ta were grown in RPMI-1640 medium at 37 °C in 5% CO₂-humidified atmosphere. RPMI-1640 medium was supplemented with 10% FBS, 2 mM L-glutamine, 100 µg/ml streptomycin, and 100 U/ml penicillin. When reaching 80-90% confluence, cells were detached by Trypsin-EDTA solution, subcultivation ratio was from 1:3 to 1:6.

2.2.5. In vitro study

To demonstrate cell growth on hydrogel surface in vitro hydrogel samples were placed into 24-well plates and seeded with BJ-5ta fibroblasts (20,000 cells per each sample). The cell growth was observed by light microscopy within 7 days.

2.2.6. Animal experiments

All studies were conducted in an AAALAC accredited facility in compliance with the PHS Guidelines for the Care and Use of Animals in Research and were approved by the institutional Animal Care and Use Committee. Six week old BDF1 mice (n = 6) were purchased at vivarium of N.N. Blokhin Russian Cancer Research Center. The mice were housed with free access to food and water. Pre-implantation procedures were carried out under strict sterile conditions. Scaffolds were immersed in a 0.9% sodium chloride solution at 37 °C for at least 30 min. Prior to implantation, animals were anesthetized using a single intraperitoneal injection of Zoletil (20 mg/ml, 50 µl per animal, Virbac SA, Carros, France) and Rometar 2% (20 mg/ml, 10 µl per animal, Spofa, Czech Republic). Subcutaneous pockets (side length 15 mm) were formed on the back of mice, and implanted scaffolds were placed on the back muscle fascia. Then the pockets were closed with non-absorbable monofilament polypropylene suture (Prolene 4.0, Ethicon). At the end of experiment animals were sacrificed by cervical dislocation. Immediately after the sacrifice, scaffolds were collected and fixed in 4% paraformaldehyde for 2 h. Then the samples were dehydrated, embedded in paraffin, and cut into 3 µm thick sections. The alternate sections were stained with Hematoxylin and Eosin according to the manufacturer protocol. Three images of each animal were randomly selected to evaluate the tissue response to the implanted scaffold. ALEICA DM4000 B LED microscope, equipped with a LEICA DFC7000 T digital camera and LAS V4.8 software (Leica Microsystems, Switzerland), was used for the sample imaging and examination.

3. Results and discussions

Conjugation of GMA with HA was performed to acquire HA derivatives capable of crosslinking. Conjugation of GMA can be carried out via reaction mechanisms of reversible transesterification or irreversible epoxy ring opening with carboxyl or hydroxyl moieties of HA (Figure 1). According to the work [4] products of latter mechanism are more stable and tend to accumulate during longer periods of time substituting products of former mechanism. On the basis of these considerations HAGM was produced as described in work [4].

![Figure 1. Reaction of HA-GMA synthesis.](image)
The original method [4] was changed in order to investigate correlation between DS and reaction conditions such as catalyst type, HA:GMA ratio and reaction time. The ability of DS regulation enables the control of crosslinking degree and, correspondingly, mechanical and biodegradability properties of scaffolds. Thus, quantitative analysis of double bonds in HA derivatives was needed. In this work we used KMnO₄ and absorption spectra measurements to easy and rapidly quantify DS in HAGM instead of using NMR.

Permanganate ions oxidize double bonds in HAGM, changing the solution color from purple to brownish yellow (Figure 2 (a)). Total disappearance of peaks at 525 nm [5] confirms a complete consumption of permanganate ions in the course of reaction (Figure 2 (b)).

![Figure 2(a,b)](image)

Figure 2(a,b). (a) Color change of KMnO₄ standard solution with different amount of added 0.5% GMA solution. (b) Decrease of KMnO₄ peaks under GMA solution addition, spectrophotometric data.

The concentration of the consumed KMnO₄ corresponds to the concentration of double bonds. After that quantity of different HAGM samples needed to reduce 1 ml of standard KMnO₄ solution were determined and their DS was counted. Results are given in Table 1.

| Changing parameter | HA:GMA, m/m | HA:catalyst, m/m | Time of reaction, days |
|--------------------|-------------|------------------|------------------------|
|                    | 1:6         | 1:10             | 1:15                   | HA:TEA | HA:TEAB | HA:TEAB | 1 | 2 | 3 | 4 | 5 | 6 |
|                    | 1:4,5       | 1:1              | 1:2                    |
| DS, %              | 10          | 13               | 22                     | 22     | 41     | 45      | 17 | 21 | 28 | 40 | 38 | 38 |

Augmentation of GMA concentration and substitution of TEA by TEAB result in higher DS. Further increase of TEAB feebly affects HA-GMA DS. Experiment with reaction time indicated that DS achieved maximum of 40 % on 4-th day and then remained the same with insignificant changes.

The synthesized HAGM has been utilized to produce hydrogel films. For this aim the initial PCC was placed between two microscope slides. The distance between glasses was adjusted using silicon spacers to 150 µm. The exposure was performed for 12 minutes, then the sample was rotated and irradiated again from another side. Afterwards the microscope slides were gently separated and the produced crosslinked hydrogel film was placed into PBS for 7 days. Aiming to demonstrate the printability of hydrogels we produced hydrogel scaffold using 3D extrusion printing as described in [3], but irradiation wavelength was changed to 365 nm. The scaffold produced with 2 mm period of grating is presented in Figure 3.
Recently, we used extract-based MTT assay to demonstrate that HAGM-based hydrogel was not toxic to cells \textit{in vitro} [3]. In the current research, cell attachment to the hydrogel scaffolds \textit{in vitro} as well as hydrogel biocompatibility \textit{in vivo} were studied. For \textit{in vitro} experiments, immortalized human fibroblasts BJ-5ta were used as model cells since fibroblasts play the leading role in tissue repairing. It was demonstrated that fibroblasts were able to grow on the hydrogel surface, confirming high \textit{in vitro} biocompatibility of the HAGM-based hydrogels (Figure 3). It should be noted, that fibroblasts preferred to attach to the rough regions of hydrogels that usually occurred at the edge of the samples.

![Figure 3](image)

\textbf{Figure 3.} HAGM hydrogel scaffold produced by 3D extrusion printing. Scale bar 2 mm.

For \textit{in vivo} experiments, scaffolds were implanted into mice (back muscle fascia) for 1, 2, and 4 weeks. Then the scaffolds within the surrounding tissue were collected, and the scaffold-induced inflammation, neovascularization, and fibrosis were evaluated. Thus, moderate infiltration of lymphocytes, granulocytes, and macrophages was found in a week after implantation, which indicated a weak inflammation process. Additionally, a minimal capillarity was noted, and the scaffold closely adhered to the tissue. It should be also noted that the scaffold thickness increased compared to the initial one (before implantation), which may be explained with hydrogel swelling due to enzymatic hydrolysis [6].

By the 2nd week the inflammation decreased, and granulation tissue was formed at the scaffold site (Figure 5 (a)). The scaffold fragments separated from the main mass were subjected to macrophagial resorption. Basophilic color in H&E staining indicated the presence of HAGM in the scaffold composition. The scaffold thickness was restored to the initial one. Finally, by the 4th week, separated fragments disappeared, and a very thin mature connective tissue capsule formed around the scaffold. No tendency to scarring was found. The scaffold thickness did not change, and the newly formed capillaries and fibroblasts did not penetrate the scaffold (Figure 5 (b)).

![Figure 4](image)

\textbf{Figure 4.} Human fibroblasts BJ-5ta growth on the hydrogel surface, 1 and 4 days of incubation. Scale bar 100 µm.
Figure 5(a,b). (a) Histological overview of a H&E stained section from mouse skin, 2 weeks after scaffold subcutaneous implantation (x100). Granulation tissue formation, scaffold fragments are surrounded with thin connective tissue capsule. An insert: macrophagial resorption of the implanted material. Large macrophages and giant cells are shown. Edema accompanied with lympho-macrophagial and weak neutrophil reaction is observed. (b) Histological overview of a H&E stained section from mouse skin, 4 week after scaffold subcutaneous implantation (x200). Scaffold preserves the initial shape. Formation of a relatively thin mature connective tissue capsule around the scaffold is shown.

4. Conclusion

In present study we developed approach to the controlled modification of sodium hyaluronate with GMA. We demonstrated easy and rapid quantitative analysis of DS in HAGM using KMnO₄. Modified HA can participate in radical photoinduced crosslinking and possesses a great potential for hydrogel scaffold production. According to in vitro data, in vivo results demonstrated high HAGM-based hydrogel biocompatibility without wound healing disorders, hypertrophic scars formation, or long-term inflammatory reactions. Furthermore, controlled introduction of double bonds can enable regulation of swelling and biodegradation rate of final hydrogel product already at the stage of preparation of PCCs for 3D printing.

5. Acknowledgements

The reported study was funded by RSCF according to the research project № 17-19-01416.

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