SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Synthesis and characterization of hydrogel and nanogel systems for dental drug delivery

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The Examination takes place at the Lecture Room of 210, Faculty of Dentistry, University of Debrecen, at 11.00. 17th October, 2016.

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, at 13.00. 17th October, 2016.

INTRODUCTION

Hydrogels are hydrophilic 3D polymer structures that can absorb high amounts of water. Due to porosity and capability for swelling they are soft and elastic, similarly to tissues, so they can be used for medical devices successfully. They are used for contact lenses or for the regeneration of skin or bones, but they can be appropriate for reconstruction of articular and cartilage alone or combined with different drugs. Nowadays the importance of the application of hydrogels in the formulation of biologically active molecules is continuously growing. Hydrogels are among the most intensively investigated materials in the field of drug encapsulation and controlled release development.

In the international literature a number of monomers and monomer systems have been studied but because of the large number of possibilities and the appearance of new materials and new properties research is still going on unabated today. The old, well known materials, when combined with new opportunities, could turn out to be entirely different and better suited for the changed modern demands. This was the course that leads from using synthetic polymers to the application of biocompatible materials under biogenic conditions. This is the road that led to developing biodegradable materials. These materials are tolerable for the organism

and can decompose into their components, and these constituents do not cause any toxic effects.

These biocompatible and biodegradable material systems are already in the application stage. Without being exhaustive, we have known e.g. PerioChip® from Periodontology, as a biodegradable chlorhexidine gluconate filled system, or Atrisorb® as an antibiotic (Doxycycline) release product. Arestin® is a newly developed minocycline containing material, where the polymeric carrier is in microbead form, which can help in the more precise control of the release. The surgical use of biodegradable polymers for the merging of wound edges or their application as bone replacement materials is well known. Several products endeavor to use the possibilities of natural or synthetic gels for the controlled release of biologically active molecules. These concepts are applied in the cases of Bio-Oss Collagen®, GEM 21S® or Easy-Graft™, where the drug release is coupled to solid graft materials. In addition we can find products, whose/in which the release of biologically active components is regulated by a collagen matrix hydrogel. These are commercially available as INFUSE® Bone Graft for (rhBMP-2) or OP-1® for (rhBMP-7). They were approved by the FDA in 2002 and 2001 respectively.

OBJECTIVES

The aims of our studies were to create different crosslinked polymer systems with dental practice used "blue light" (385-515nm) photopolymerization, and use these for controlled release of dental drugs. Our aims were:

With the use of HEMA-PEGDMA components:

- Creation of hydrogels with different crosslinking density
- Synthetization of nanoparticles, and using them for nanocomposite hydrogels
- Investigation of mechanical parameters of the gels
- Study of the release properties of the hydrogels

With the use of poly-γ-glutamic acid (PGA):

- Synthetization of dental practice used "visible-light" (385-515nm) photopolymerizable polymer and nanoparticle
- Use of these polymer and nanoparticle as stock materials for hydrogel and nanogel creation
- Study of mechanical parameters of the gels
- Determination of biocompatibility
- Investigation the effects of the gels on cells by different microscopy methods
- Description of swelling abilities and release properties of the hydro- and nanogels

MATERIALS AND METHODS

Synthesis and characterization of HEMA-PEGDMA hydrogel, nanoparticle and nanocomposite

In the first part of our work we created biocompatible nanoparticles (NPs) by micellar polymerization. The reaction mixture was continuously stirred by magnetic stirrer under nitrogen atmosphere. The components of the organic phase were 2-hydroxiethyl methacrylate (97%, Sigma-Aldrich Ltd.) (HEMA) monomer, poly (ethylene glycol) dimethacrylate (Mn: 550) (PEGDMA) (Sigma-Aldrich Ltd.) crosslinker and n-butyl-alcohol (Spektrum 3D Ltd.). The hydrophilic part consists of sodium lauryl sulfate (Spektrum 3D Ltd.) (SLS) in solution of potassium persulfate (98%, Reanal Co.,) (KPS) as thermo-initiator in concentration of 2.4%, and N,N,N',N'-tetramethyl –ethylene diamine (99%, Sigma-Aldrich Ltd.) (TEMED) as catalyst.

The composition of the biocompatible hydrogel was HEMA and PEGDMA in various ratios (90:10, 75:25, 50:50, 25:75, 10:90). The nanocomposite hydrogel (NCHGs) consists of HEMA:PEGDMA 50:50 matrix hydrogel and nanoparticles with the same composition. Anthraquinone-2-sulfonic sodium salt (~99%, Fluka AG. Buchs SG) was applied as photoinitiator. The reaction time of the photopolymerization was 20 minutes using a

Kulzer Palatray (435 nm, ~0.1 watt/cm^2) photopolymerization lamp.

The sizes of the NPs were determined according to the hydrodynamic diameter by dynamic light scattering (BI-200SM, NbYAG solid state laser) (DLS). In the cases of NCHGs the broken surfaces were imaged by scanning electron microscope (HITACHI S4300) (SEM).

The mechanical properties of NCHGs and hydrogels in different crosslinking densities were compared by INSTRON 4302 (load cell: 0.1 kN, compression: 2 mm/min). This analysis followed the rules of MSZ EN ISO 604:2003. The results were investigated by Post-Hoc Test.

The swelling ability of the gels was determined by mass measuring. The weight of swollen gels was compared with the weight of the gels after the polymerization. For the study of the release properties of the gels chlorhexidine gluconate (20% solution from Spektrum 3D) was used as a generally administered drug in dentistry. The amount of drug released was measured by Merck-Hitachi LaChrom HPLC machine with C18 Nucleosil (5 μm) column.

Preparation and characterization of PGA-hydrogel

In the second part of our work hydrogel was synthetized from biodegradable poly-γ-glutamic acid (PGA)

(M_W=1.2×10^6, GPC) that was biosynthetically prepared in our lab. Water-soluble 1-[3-(dimethyl amino) propyl]-3-ethylcarbodiimide hydrochloride (EDC) (Sigma-Aldrich Ltd) was used to increase the reactivity of the carboxyl groups. Methacrylating agent 2-aminoethyl methacrylate hydrochloride (90%) (Sigma-Aldrich Ltd.) (AEM) was applied to create reactive groups necessary for photopolymerization. The reaction solution was mixed intensively for 24 hours. The successfulness of the reaction, the binding of the reactive groups was proven by nuclear magnetic resonance method (NMR) (Bruker 200SY).

From the methacrylated-PGA (MPGA) hydrogel was created using Irgacure 2959 (~99%, CIBA) in a KULZER Dentacolor XS (435 nm, ~10 watt/cm^2) photopolymerization chamber.

The mechanical analysis was performed using an INSTRON 5544 according to the method described earlier.

For the release study, metronidazole as a more modern drug was chosen. The amount of drug released was measured by Waters 600 HPLC machine with Nucleosil (C18, 5 μm) column.

The toxic effect of the gels on cells was investigated by MTT and LDH tests, and the morphological changes of (HaCaT) keratinocyte cells were followed. These experiments proved the applicability of the gels in biological systems.

Synthesis and characterization of PGA-based nanoparticles and nanoparticle-based hydrogel (nanogel)

In the final part of our work, PGA nanoparticles were synthetized by crosslinking PGA from a commercial supplier (Nanjing Saitaisi Biotechnology Co. Ltd.) (M_W= >1×10^6Da). After an EDC carboxyl group activation crosslinks were formed by 2,2'-(ethylenedioxy)bis(ethylamine) (98%) (Sigma-Aldrich) (EDA) as crosslinker, while the reaction solution was intensively stirred. In a second step methacrylation reaction was performed for the creation of the photopolymerizable groups, as it was described earlier.

Verification of the reaction, determination of particle size, creation of the nanogels, mechanical investigations and swelling ability study were performed as these were described in the earlier parts.

The release measurements were done with ampicillin (Sigma-Aldrich), on a Dionex Ultimate 3000 HPLC machine, using an Accucore^{TM} aQ (C18, 2.6 μm) column.

Alamar Blue test was performed to assess basic cell viability, and the effects of the nanogels on the SAOS2 (osteosarcoma) cells were investigated by fluorescence- and confocal laser scanning microscopy.

RESULTS AND DISCUSSION

Synthesis and characterization of HEMA-PEGDMA hydrogel, nanoparticle and nanocomposite

In our first experiments both the DLS and SEM results have proven that the size of the 50/50 HEMA/PEGDMA NPs was around 100 nm or less. When the particles were dried, the SEM images presented sizes between 50-150 nm, and the DLS results showed size distributions between 5-500 nm. The 50/50 HEMA/PEGDMA hydrogels were modified with these NPs. This NCHG contained 30% of particles. The mechanical properties of the hydrogels in different crosslinking densities (10-90%) were compared with this NCHG. It was found that the increase of stress was faster in the case of NCHG than the 90% crosslinked hydrogel, but the compression strength was not higher. We can declare that the application of NP greatly influenced the mechanical properties, and considerably increased the stiffness of the hydrogel. Considering the swelling abilities, the base hydrogel reached the equilibrium weight in the first half an hour, while this was 22 hours in the case of NCHG. There was a difference in weight gain also, because it was 13% for the base hydrogel and 21% in the case of the composite. The release properties, as a central part of our studies, showed that the use of NPs influenced the

features of hydrogels. In our experiments when either the matrix or the NPs were loaded with drug, the other, unfilled component cancelled the change contributed by the filled component. When the nano- and matrix part were both loaded with drug a certain prolonging effect was observed in the release in the first 48 hours.

Preparation and characterization of PGA-hydrogel

After the study of the biocompatible model, we wanted to create a biodegradable, photopolymerizable, controlled drug delivery system. In the first step for this aim the PGA was modified by methacryloyl-group. The successfulness of the reaction was proven by NMR method, and spectra showed that instead of the calculated 50%, the methacrylation-rate was nearly 10%. However, this amount of reactive-groups was enough to reach the 90sec polymerization time using a new initiator. The mechanical analysis showed that the value of compression strength and strain were 12.99 N (SD:5.14) and 0.94 mm/mm (SD:0.16) respectively. These values meant correspond to a compression stress value of 0.77 MPa (SD:0.27) MPa and a Young modulus of 0.36 MPa (SD:0.06). These values describe a structure that is stable and highly elastic. The swelling kinetics of drug-loaded and unloaded hydrogels were investigated in parallel by mass measurement. We observed significant weight gains in the first half hour, 150% in the case of unloaded, and

200% for loaded gels. The rate of swelling was decreased in the second hour: the unloaded gels reached a level over 200% while metronidazole-loaded gels were close to 300%. By the 4th and 5th hours the gels reached an equilibrium state at around 250% and 310%, respectively. After the investigation of the swelling profiles we conducted a study of release properties. The measurements were performed in five parallel experiments, in the case of loaded gels the drug content was 3.33 mg/g metronidazole/hydrogel. The dynamic of the release process followed the swelling. In the first two hours an "initial burst effect" was observed, where the amount of drug released reached 5 ng/mm^2. After this the system achieved an equilibrium state in the next 6 hours.

Demonstration of the biocompatibility of this biodegradable drug delivery system was essential for further applicability. This was investigated for the stock polymer (10-40 mg/ml), photoinitiator (0.25-2.5 mg/ml) and prepared hydrogel by MTT and LDH tests on CaCo2 cell line. Furthermore cell morphology investigations were done on HaCaT cells. Our results showed that none of the materials affected the viability of the cell lines used; the maximum decrease in viability was 2%. In the same concentration ranges the cytotoxicity test gave similar results: the maximum value was less than 4% for stock polymers or prepared hydrogels, and it was less than 5% for the initiator. The cell morphology investigations showed that the HaCaT cells were attached

to the control coverslips and MPGA hydrogel surfaces, and the cells did not exhibit significant visual differences in morphology. Based on the 6- and 24-hour observations we concluded that the modified polymer (MPGA) based hydrogel is biocompatible, and it does not alter the normal keratinocyte morphology.

Synthesis and characterization of PGA-based nanoparticles and nanoparticle-based hydrogel (nanogel)

In the final part of our work nanoparticles (NPs) were formed from the biodegradable PGA by an intermediate modification. Subsequently, reactive groups were coupled to the NPs – by the method described earlier – in order to enable the NPs to photopolymerize. NMR spectra showed the success of the reactions. The peaks of the methacryloyl-groups and the crosslinker (diamin) were clearly identified in the spectra. The size determinations of prepared stock materials were done by SEM and DLS methods. The preparation of broken and superficial surfaces occurred after careful dehydration by critical point drying. The SEM images clearly show the different structures under 100 nm which built up the whole structure. The sizes of fibers and particles are between 50 and 100 nm. The results of DLS analysis – in native aqueous medium - corresponded well with the SEM pictures. We can see that the objects around or

under 100 nm create the major part of the particles. Using this methacrylated-PGA-NPs and Irgacure 2959 (2n/n%) as photoinitiator, hydrogel/nanogel was polymerized in 3 minutes. The mechanical parameters of this nanogel were a Young-modulus of 0.37 MPa (SD:0.20) and a compression stress value of 0.38 MPa (SD:0.10), while the maximum strain was 85%. These values show that this nanogel is not as strong as the MPGA-hydrogel, but these physical properties make it strong enough to be appropriate for practical use. The swelling ability of the nanogel decreased compared to the simple hydrogel, the value of this is around 110%. This property could be beneficial in practice, because the space next to a tooth is limited. The widely used antibiotic ampicillin was chosen as the drug to be loaded into the nanogel for the investigation of release properties. The release study of the drug-loaded nanogel was measured by cumulative method, in one week period; saline solution was used as leaching medium. The HPLC results showed - the earlier mentioned - initial "burst effect", and after that the equilibrium was reached by the 24^{th} hour. The main difference between the hydrogel and nanogel was that the released drug in the case of nanogel remained on the level of 80%. This retaining effect could be the basis of a control possibility. The biocompatibility of the nanogels was investigated by Alamar Blue test. The viability and proliferation rates were measured by SAOS-2 cell line morphology tests. Furthermore, confocal laser scanning

and fluorescence microscopy images proved the biocompatibility of the prepared nanogels.

SUMMARY

In the first part of our research we created hydrogels with various crosslinking densities from biocompatible (HEMA, PEGDMA) materials and we characterized the mechanical properties of these materials. From the same materials we prepared nanoparticles, and using these components nanocomposite-hydrogels were synthetized. The photopolymerization of hydrogels and nanocomposite were done with "visible light" (385-515 nm) that is used in dentistry. The compression strength of the nanocomposite-hydrogel (50% crosslinked hydrogel was modified with 50% pre-crosslinked nanoparticles) was higher than the 90% crosslinked simple hydrogel. Study of the release properties showed that the application of nanoparticles can considerably influence this characteristic. In the case of the basic hydrogel the release reaches the maximum by the 6^{th} hour, while the use of nanoparticles causes sustained release until the 48^{th} hour.

In the second and third parts of our work a biodegradable PGA was modified to create materials with photopolymerizable properties for to be used in dental practice. The modification by reactive

methacryloyl-groups resulted in photopolymerizable polymer for hydrogel. The pre-crosslinked and methacrylated PGA-polymer – photo-reactive nanoparticle – was used as base material for nanogels. Applying an appropriate photoinitiator the reaction time was 90-180 sec. The mechanical properties and swelling abilities of the prepared hydrogels and nanogels fulfill the requirements of the application area – in the mouth e.g. next to a tooth on an inflammatory area.

The biocompatibility studies proved that neither the polymerized gels, nor the raw materials have a cytotoxic effect in MTT- or LDH-tests. The cell morphology investigations of HaCaT cells gave similar results after 6- and 24-hour observations. In the case of nanogel the biocompatibility was proven by Alamar Blue test, and this result was confirmed by fluorescence and confocal laser scanning microscopy images. The biocompatibility test showed the viability of cells in a 3-day interval, while in the case of the microscopy methods we could follow the proliferation tendency of SAOS-2 cells during a one week period. All of the results proved the biocompatibility of the cells, and showed that these gels are suitable to provide the appropriate conditions for proliferation.

The release properties of hydrogels showed that the leaching of the antibiotic drug metronidazole was fast under the conditions of the experiment, taking a few hours. This could be changed in the future by combining

the gels with other components. Release dynamics could also be influenced by different conditions at the place of application. In the case of nanogel the release of ampicillin showed a certain (~20%) retaining effect, compared to hydrogel. This effect could create the basis of a control possibility.

In summary, these hydrogel and nanogel systems could be regarded as potential candidates for biomedical applications e.g.: intraorally for treatment of periodontal diseases, or in surface modification for improving the integration of implants. Using these materials as in situ polymerizable drug delivery systems could be beneficial, and could serve as a foundation for further research.

NEW SCIENTIFIC ACHIEVEMENTS

The use of HEMA-PEGDMA as base-materials:

I. Hydrogels - in different crosslinking density -, nanoparticles, and „nanocomposite-hydrogel" were synthetized.
II. The reinforcing effect of the nanoparticles on the hydrogel was proven by analysis of mechanical properties.
III. A controlling effect of the nanoparticles was shown in the release properties in the case of nanocomposites.

The use of PGA as base-material:

I. Dental practice used "visible-light" (385-515nm) polymerizable material was created, and was used to create hydrogel by photopolymerization.
II. "Visible-light" polymerizable PGA-nanoparticles (MPGA-NP) were created in a two-step reaction.
III. Hydrogel/nanogel consisting exclusively of MPGA-NP was created by "visible-light" polymerization that is used in dentistry.
IV. The applicability of PGA based hydrogel and nanogel as drug delivery systems was proven by their physical properties and biocompatibility.

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Subject: Ph.D. List of Publications

Candidate: József Bakó
Neptun ID: SG9XWE
Doctoral School: Doctoral School of Dental Sciences
MTMT ID: 10036858

List of publications related to the dissertation

1. **Bakó, J.**, Kerényi, F., Hrubi, E., Varga, I., Daróczi, L., Dienes, B., Csernoch, L., Gáll, J., Hegedűs, C.: Poly-[gamma]-Glutamic Acid Nanoparticles Based Visible Light-Curable Hydrogel for Biomedical Application.
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Total IF of journals (all publications): 12,444
Total IF of journals (publications related to the dissertation): 5,759

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

16 June, 2016

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1. **Bakó József**, Hegedűs Csaba, In situ –kék fény hatására-polimerizálható nanogél előállítása, és fogászatban alkalmazható hatóanyag-leadó tulajdonságának vizsgálata, Árkövy Vándorgyűlés, Szeged, 2016. május 5-7.
2. **Bakó József**, Hegedűs Csaba, Látható fény hatására polimerizálódó, poli-γ-glutaminsav nanorészecskékből felépülő nanogél, mint hatóanyag leadó rendszer. Fogpótlástani Napok, Pécs, 2015. Szeptember 24-26.
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6. **Bakó József**, Ujhelyi Zoltán, Kovácsné Bácskay Ildikó, Borbíró István, Biró Tamás, Borbély János, Hegedűs Csaba, Látható fényre polimerizálódó, biodegradábilis hatóanyagleadó rendszer előállítása és vizsgálata, XIV. Debreceni Fogászati Napok, Debrecen 2013. Április 12-13.

7. **Bakó József**, Ujhelyi Zoltán, Kovácsné Bácskay Ildikó, Borbíró István, Biró Tamás, Borbély János, Hegedűs Csaba, Fogászatban alkalmazható hatóanyagok leadására képes, fényre polimerizálódó hidrogél rendszer előállítása és jellemzése, Pécs, Árkövy Vándorgyűlés, 2012. Szeptember 20-22.
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15. Szepesi Márta, **Bakó József**, Borbély Zsuzsa M., Veress Adrienn Judit, Borbély János, Hegedűs Csaba, Hatóanyag leadására alkalmas hidrogélek előállítása és vizsgálata, Tudományos továbbképző konferencia és fogorvostalálkozó, Szeged 2006. Április 22.
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