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Hydrogels based on polysaccharide-calcium phosphate with antibacterial / antitumor activity for 3D printing

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Abstract. The purpose of this study was to develop hydrogels for 3D printing of sodium alginate /gelatin /octacalcium phosphate-based constructs with antibacterial and antitumor activity intended for bone defects replacement in patients with malignant diseases. In this work, we evaluated the drug release kinetic and physico-chemical characteristics of constructs, as well as their specific activity, biocompatibility and osteoplastic properties by means of *in vitro* and *in vivo* tests. The principal possibility of creating the biocompatible bone substitutes with antibacterial /antitumor activity and osteoconductive-retaining properties of 3D printing method was demonstrated.

1. Introduction

Many resent concepts for osteoplastic materials investigations are inspired by the structure of natural bone tissue which extracellular matrix comprises a perfect nanocomposite consisted of collagen fibrils chemically linked to carbonated apatite. In this term, different biomimetic approaches involving natural polymers and calcium phosphate (CP) ceramic were developed in last decades [1, 2]. In our study, we used biocompatible polymers—sodium alginate and gelatin in combination with octacalcium phosphate (OCP)-based ceramics. Being a precursor of biological apatite OCP promotes a mineralization of new bone tissue and also demonstrates convenient solubility in simulating body fluids [3, 4].

At the present time, a number of requirements have been postulated for biomaterial: biocompatibility, high surface area, high degree of porosity, optimal rate of resorption (biodegradation) *in vivo*, corresponding to the rate of new bone formation, osteoconductive and, more preferable, osteoinductive properties. Recent technologies of rapid prototyping, e.g. 3D printing, are capable for creation of personalized grafts with precise geometry of defects according to patient's CT/MRI data [5]. On the other hand, the functionalization of three-dimensional printed structures for local delivery of antitumor drugs or antibiotics can theoretically increase the effectiveness of such therapy by improving the accumulation of the drug in bone tissues, reducing its dosage and toxicity without significantly increasing the duration of bone regeneration [6].

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The study was devoted to hydrogels based on polysaccharide-calcium phosphate with antibacterial / antitumor activity for 3D printing of constructs for bone defects replacement. Thus, we studied effects of the hydrogel composition on resulting structure, porosity and mechanical properties of 3D printed constructs. A complex composition of constructs indicated to the possibility of their multipurpose use: not only as a "simple" implant material, but also as a bioconstruction containing biologically active molecules (growth factors) or drugs.

2. Materials and methods

An experimental three-dimensional constructs (3DC) was produced according to original technology (Patent RU2606041C2) using a custom-designed injection 3D printer. The hydrogel ("ink") for 3D printing consisted of sodium alginate, gelatin, and octacalcium phosphate in proportion of 56/14/30 wt. %. The functionalization of constructs with vancomycin (3DCVanc) or doxorubicin (3DCDox) we performed by addition of drugs to the initial dry components of hydrogel. The process of 3D printing was as follows. The hydrogel having viscosity 10–29 Pa was placed in disposable syringe of 3D printer and then injected through a needle onto the precooled (–20°C) surface with alcohol and calcium chloride water solution (70/30) filled bath. The printed semi-solid constructs were freeze-dried at –50°C and 6 × 10–5 atm. for 10–12 h. Solidified 3DCs were washed in distilled water and dried at 37°C for 24h to finalize the synthesis of nonorganic components.

Scanning electron microscope (SEM) Vega II (Tescan, Czech Republic), working in secondary and backscattered electron modes, was used for microstructure studies. The samples were sputter-coated with a 25 nm thick gold layer prior imaging to impart electrical conductivity of the surfaces. FTIR spectroscopy was carried out with Nicolet Avatar system (Thermo Fisher Scientific, USA) using the powder mixtures of KBr samples.

Compression testing was carried out using an Instron Electro Puls E3000 testing machine (Bucks, UK) operating at a crosshead speed of 1 mm min^{-1}. The 3D printed cylindrical samples for testing were about 12 mm in height and 6 mm in diameter.

Biocompatibility of three-dimensional constructs was evaluated on the model of their subcutaneous implantation of male BDF1 mice (Scientific Center for Biomedical technologies of FMBA of Russia). The experiment implied 4 groups of 12 animals formed according to the number of functionalized compositions of implantable 3D constructs and 1 control group. Thus, 4 types of 3DCs saturated with 30 or 70 wt. % vancomycin (3DCVanc30 and 3DCVanc70) and 3 or 6 wt. % doxorubicin (3DCDox3 and 3DCDox6) were tested. Animals were carried for 2, 4, 8, 12 weeks after surgery. At the indicated time, two animals from each group were withdrawn from the experiment. Samples of materials were extracted and observed with stereomicroscope equipped with a digital video camera (Olympus, Japan), then fixed in a 10% formalin solution and embedded in paraffin. Histological samples (a thickness of 4 um) were stained with hematoxylin-eosin and documented with Eclipse Ti microscope and digital camera DS-Fi1c (Nikon, Japan).

3. Results

Basically, our approach for the production of 3D constructs based on polysaccharide-calcium phosphate involved the prepeartion of the original hydrogels into which the required concentrations of antibacterial (vancomycin) and antitumor (doxorubicin) drugs were added at the stage of dry components mixing. Using capillary viscosimetry, it was found that the administration of antibiotic in concentrations of 30 and 70 wt. % and antitumor preparation in concentrations of 3 and 6 wt. % did not significantly affect the values of viscosity and hydrogel fluidity, allowing the providing of 3D printing. According to SEM data, 3DCVanc maintained its porosity and tabular structure in the presence of 30 wt. % vancomycin. Increasing of concentration of vancomycin in the hydrogel slightly transformed the structure: pore size increased to 200–300 μm; there was a uniform spreading of drug on the surface of the specimens, which thickness increases proportionally to concentration of vancomycin (figure 1). Addition of doxorubicin led to serious changes in the inner structure of 3DCDox.

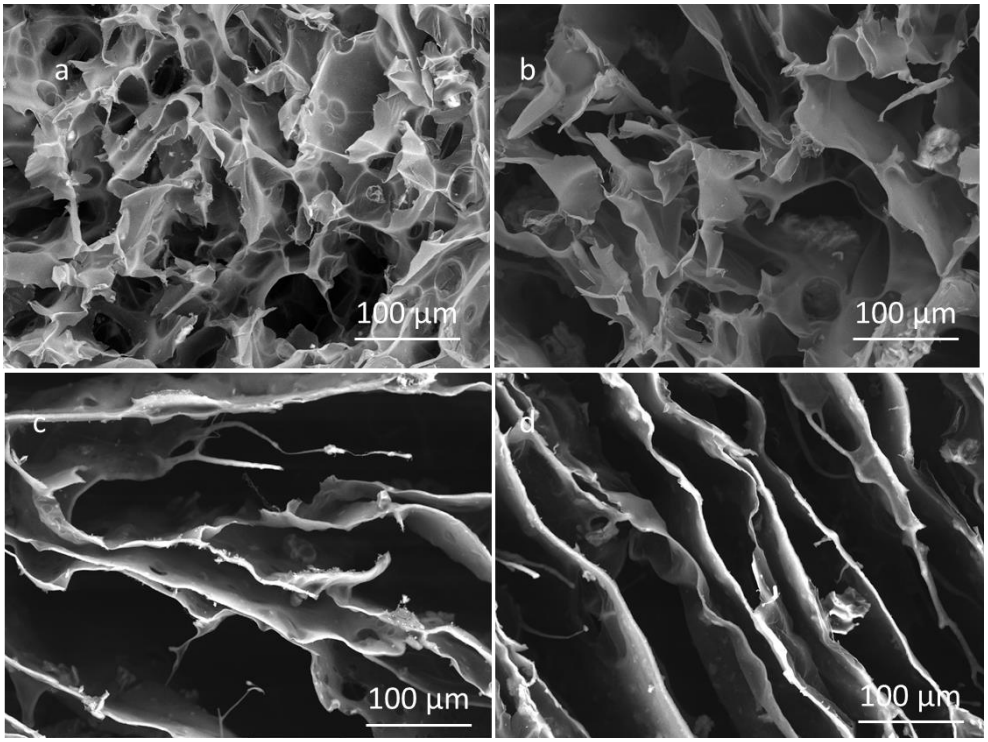

Figure 1. SEM images of microstructure of the 3DCs with 30% vancomycin (a), with 70% vancomycin (b) and 3% doxorubicin (c), 3% with 6 % doxorubicin (d).

The compressive strength of 3DCs was decreased after addition of higher drug concentrations to the hydrogel for 3D printing. The compressive strength (5.5 MPa) did not differ between 3DCVanc30 and control 3DCs. Meanwhile, its value decreased to 4.4 MPa after addition of 50 wt. % vancomycin and to 3.7 in case of 70 wt. %. For the materials comprising 3 and 6 wt % of doxorubicin the value of strength was changed more dramatically and decreased 2.5-fold compared to empty 3DCs (1.8 and 2.2 MPa, respectively). The determination of the effects on the component composition after the introduction of drugs to 3DC was determined by IR spectroscopy. In all investigated constructs, IR spectra contain bands corresponding to the ν_1, ν_2 and ν_4 modes of the phosphate groups in the range of waves 960, 1020-1120 cm^{-1} and 560-660 cm^{-1}, respectively (figure 2). The characteristic bands of vancomycin 1650, 1506, 1230 and 1047 cm^{-1} showed that antibiotic administration to the hydrogel did not lead to a change in the chemical composition of vancomycin and did not cause any noticeable change in the composition of the 3DC. The IR spectra of composite materials based on the hydrogel with introduced doxorubicin, according to the corresponding bands 1616, 1583, 1207 cm^{-1}, also confirmed the absence of a change in the chemical composition.

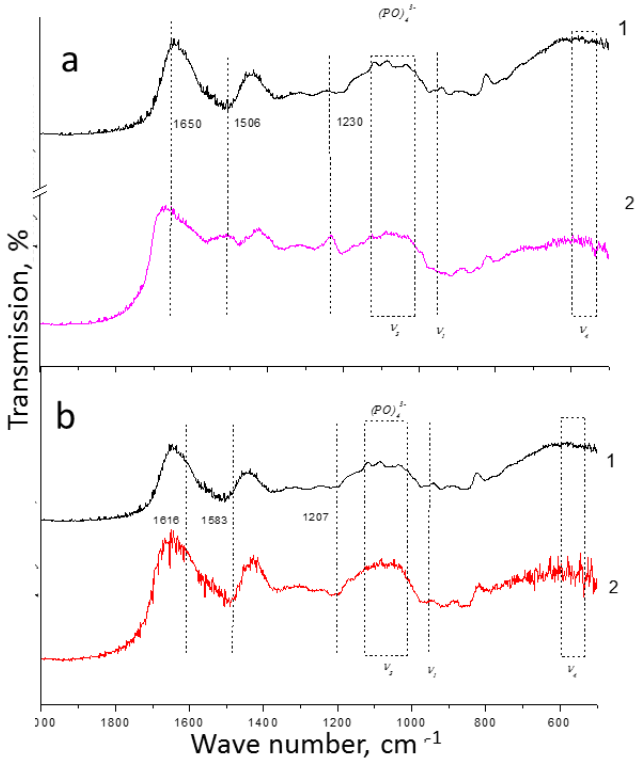

Figure 2. IR absorption spectra of 3DC based on polysaccharide-calcium phosphate – line 1 (a, b); with vancomycin – line 2 (a); with doxorubicin – line 2 (b).

In case of control printed 3DC based on sodium alginate / gelatin / OCP without medicinal drugs a thin fibrous capsule comprised connective tissue with a regular orientation of the fibers, moderate neutrophil infiltration and active vascularization could be visible in 2 weeks after subcutaneous implantation (Figure 3). Aseptic inflammation in the implantation zone substantially ameliorated in 4 weeks after the surgery. This indicated that composition of 3DC promoted the "transition" of surround tissue to the regenerative state.

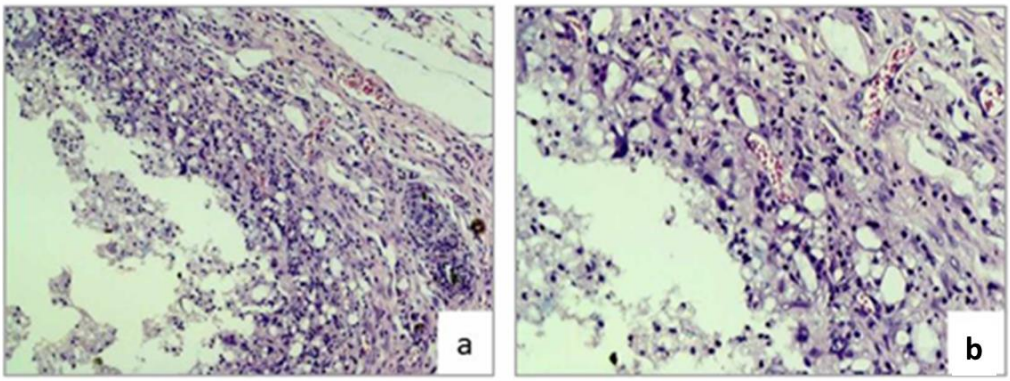

Figure 3. Images of the 3D printed samples in 2 weeks after subcutaneous implantation in mice. HE staining. Magnification: a - x100, b - x200.

After subcutaneous implantation the 3DCs saturated with antibiotic vancomycin (3DCVanc30 or 3DCVanc70) demonstrated well biocompatibility similar to those for control constructs. The signs of inflammation or necrosis were absent, and the formation of a loose connective tissue capsule even thinner in comparison with the capsule around control samples could be observed (Figure 4). Numerous foreign-body giant cells indicated the beginning of resorption processes. A rather different picture was observed when biocompatibility studying of 3DCs with antitumor drug doxorubicin was performed. In the early stages (2 weeks) after implantation the strong inflammatory reactions accompanied with intensive leukocyte infiltration developed in surrounding tissues (figure 4). There were no bioresorption signs in both polymeric and CP components of the 3DCDox composite material. The spreading of connective tissue in the inner part of implants and new blood vessels formation was hampered by the formation of thick connective tissue capsule. The dermal cells from internal layers the skin possessed cytoplasm vacuolization and apoptotic morphology. Thus, the antitumor drug released from the constructs rendered cytotoxic effect on surrounding tissues, which led to the development of aseptic inflammation and gradual incapsulation of 3DCDox. Nevertheless, the severity of the these processes allowed us to expect a powerful local antitumour effect after functionalized 3DC implantation, that can be useful in filling bone defects after surgery for primary malignant bone tumors or metastases.

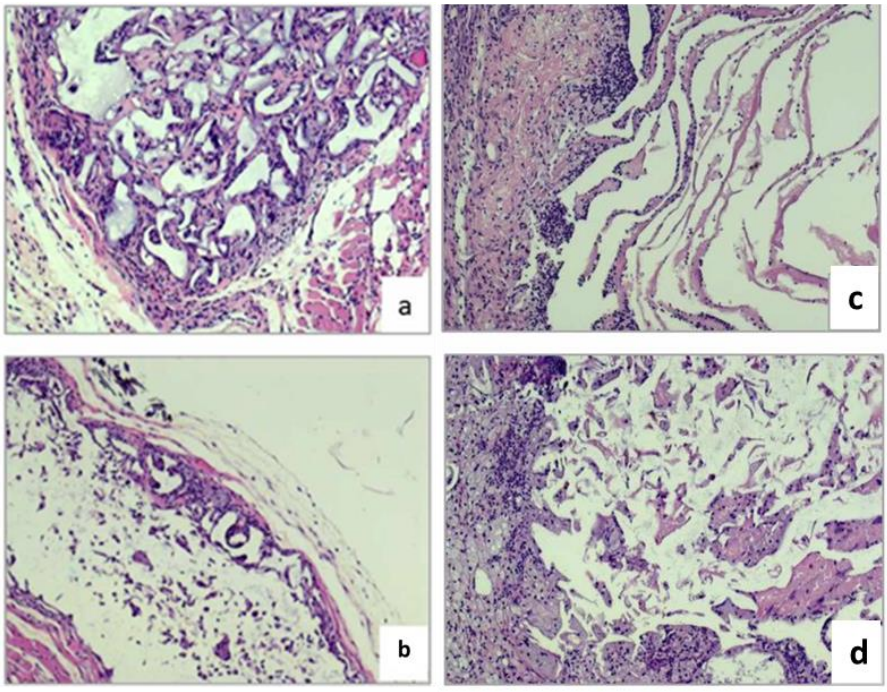

Figure 4. Images of functionalized 3DC in 2 weeks after subcutaneous implantation in mice. 30% vancomycin (a), 70% vancomycin (b) 3% doxorubicin (c), 6 % doxorubicin (d). HE staining. Magnification: x100.

4. Conclusion

The approach for 3D printing with low viscosity biopolymers and calcium phosphate components containing drugs and intended for bone defects replacement was created. Also the influence of the polymer - drug ratio on fluidity of hydrogel for 3D printing was estimated. Physicochemical characteristics of compositions based on sodium alginate, gelatin and octacalcium phosphate with

different concentrations of drugs were studied as well. It was found, that components of the system were stable during 3D printing. The functionalization of printed 3DCs did not cause dramatic deterioration of their biocompatibility properties. As a result, a complex study of physicochemical and biological features of functionalized constructs revealed that 3DCVanc and 3DCDox retain valuable features of composite bone grafts, such as porosity, phase compound and mechanical strength, but also demonstrated functional activity in vivo.

Acknowledgments
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