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Polycyanoacrylate porous material for bone tissue substitution

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It has been done the proof of concept study for design of porous biodegradable material containing nanocapsules and two actives with independent release – bimodal drug eluting implant. Completely synthetic safe material free from risk of prion and virus contamination was tested *in vivo*. The method to control the rate of biodegradation of poly-2-cyanoacrylic polymer was developed. Novel perfluorinated 2-cyanoacrylic esters have been applied for chemical modification of polyethyl-2-cyanoacrlylate copolymer. Internal imide-cycle formation has been used to retard the rate of enzymatic hydrolysis of 2-cyanoacrylic copolymer main chain.

Introduction

Two decades of intensive study of different heat resistant virus infections leaved an unsatisfactory conclusion that there is no safe way to prepare tissue substituting implants for orthopedic surgery based on biomaterials of animal and human origin[1-5]. The risk of prion and virus infection can't be eliminated even by X-ray treatment[6-8]. It causes restrictive changes in the regulatory of US and most European countries. The new reality initiates the wave of study in the area of completely synthetic, polymeric implants for bone and tissue substitution which can guarantee absence of sterilization resistant prions and other dangerous infections[9-11].

Functional requirements to polymeric implants also have been changed. Modern medical study indicates that biocompatibility and inert properties are not enough for tissue substituting products. Biodegradable implants able to be substituted with native tissue should contain a combination of actives improving both biocompatibility and controlling rate of biodegradation. At the first period after implantation, counted as several days, the key regeneration process is just wound healing and control of inflammation[12,13]. It requires the presence of specific actives located in the limited area close to the implant. At the second stage considered as degradation of polymer synchronized with its substitution by targeted native tissue the presence of morphogenesis proteins becomes essential[14-17]. In such a manner a medical device should contain at least two actives with independent rate of release considered as polymeric composite material with bimodal drug release and controlled rate of biodegradation. The third requirement is the open type porosity structure suitable for vascularization and cell immobilization during tissue substitution process[18].

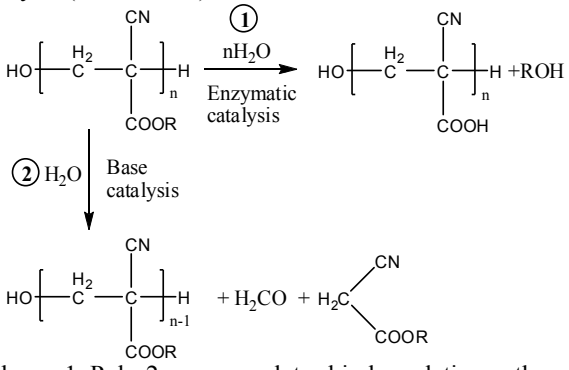
For proof of concept we developed material providing quick release of wound healing accelerator – insulin and relatively slow release of collagen – the model of morphogenesis proteins. While insulin, responsible for wound healing must release rapidly in first several days, the rate of release of collagen, responsible for tissue substitution must be rather slow. It should be admitted that choice of polymers for preparation of biodegradable materials is limited be few types.

Polylactides are the most popular chassis for manufacturing of biodegradable implants, however, it is quite poor drug carrier. The preparation of drug eluting medical devises based on it requires a complex chemical modification which often results insufficient drug capacity. Also the X-ray treatment is the only method applicable for sterilization of porous articles[19-21]. It is a meaningful shortcoming. The actives of polypeptide nature cannot be used in this case due to cross-linking and inactivation on X-ray treatment[22,23]. Alternative polymer for biodegradable implants is poly(hydroxybutyrate), however, it has the same shortcomings including slow rate of biodegradation and tough sterilization conditions[24,25].

Poly-2-cyanoacrylate based copolymers are unique materials allowing to overcome most of the shortcomings mentioned before. It is self-sterile, free from both bacterial, viral and prion contaminations, and also it is the only acrylate subjected to enzymatic biodegradation in the body[26-29].
It defines our choice to use it as a chassis for development of poly-2-cyanoacrylate based drug eluting foam material with multimodal drug release for bone substituting biodegradable implants.
In such a manner, the main goal of the research is the proof of concept study for design of porous biodegradable material containing two actives with independent release – bimodal drug eluting implant.

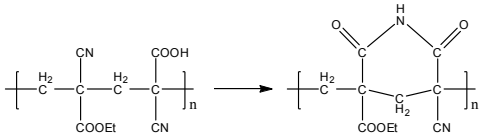
Results and discussion

The material comprises foam of open type porosity prepared from polycyanoacrylic matrix. The wall of each cell contains nanocapsules inside. It was described in terms of foam material of specific density in the range from 0.018 to 0.25 g/sm^3. Mechanical properties and degradation behavior also have been tested by methods conventional for foams. It comprises measurement of compression strength at 10% deformation, which grows with the density of foam from 0.016 to 1.10 MPa respectively and decreases on degradation of polymer. The specie is considered degraded as soon as it lost 50% of initial compression strength. The material is designed to proof the concept for tissue conducting biodegradable foam rather than for preparation of high strength polymer for bone mechanical substitution. This is the reason for modulus is nothing to compare with that of native bone tissue. However, it can be substantially increased with the growth of specific density.
Porous matrix has been synthesized from the mixture of cyanoacrylic monomers. The main component is polyethyl-2-cyanoacrylate which is widely used as surgical glue. It can degrade *in vivo* according to two mechanisms. The first one is enzyme dependant (Scheme 1-1) while the second is a common hydrolysis (Scheme 1-2).



Scheme 1. Poly-2-cyanoacrylates biodegradation pathways: 1 -α-proteolytic enzyme dependent biodegradation, 2 – β-hydrolytic biodegradation

The rate of its biodegradation in the body is too high. The time for 50% strength loss *in vitro* is in the range of 15-20 days. It must be synchronized with bone growth rate which strongly depends on numerous factors, which rather difficult or impossible to control. As soon as we cannot control the bone growth, we must control implant biodegradation. It has been done using two approaches. The first one is based on incorporation the fluorine containing biodegradation retardant into the matrix. It is 1,1,1,4,4,4-hexafluoro-2,3-bis(trifluoromethyl)butane-2,3-diyl-bis(2-cyanoprop-2-enoate) (compound B on Scheme 3). The inhibitor is a poor substrate to esterases and also the hydrofobisation and cross-linking agent. The second approach is based on partial imidization of acrylate main chain. It requires the presence of additional comonomer providing acidic moieties subjected to cyclization at physiological temperature according to the scheme 2.



Scheme 2. Imidization of 2-cyanoacrylic acid/ethyl-2-cyanoacrylate copolymer

The 10% additive of mixture of both inhibitors in ratio 1:1 (mass.) provides degradation retard from 20 days up to more than 3 months.
Bimodal drug elution was achieved by encapsulation of insulin into poly-2-cyanoacrylate nanocapsules and by covalent bonding of collagen to the matrix material. The collagen release is synchronized with biodegradation of implant while insulin elution can be described in terms of diffusion from nanocapsules.
Collagen is easily bonded to terminating groups of polyethyl-2-cyanacrylate during the process of anionic polymerization initiation, however, it is not enough. The main chain was modified by incorporation of perfluorophenyl-2-cyanoacrylate providing high capasity multi-site quantitative bonding of proteins according to the Figure 1. It is the variant of the well-known method of activated esters.

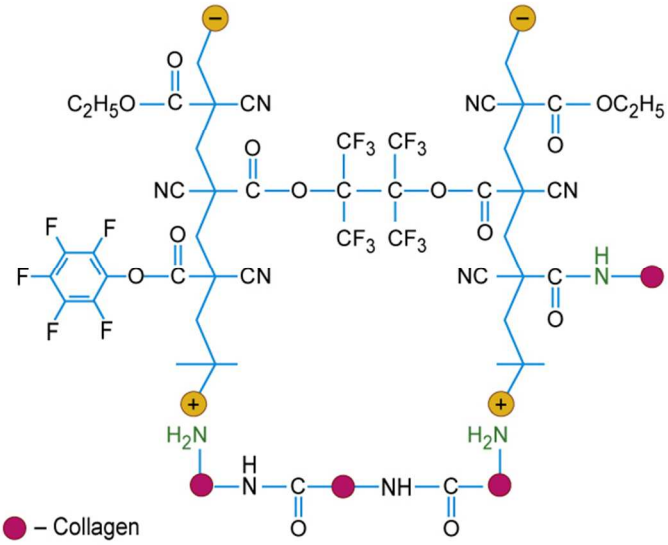
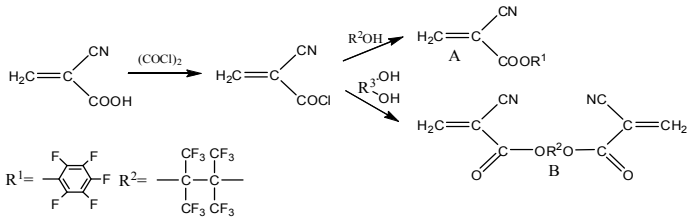


Figure. 1. Key steps of copolymer chemical modification

Introduction of collagen into the material significantly drops mechanical properties of the foam and increases rate of its biodegradation. Actually 30% additive is a maximum which keeps acceptable compression strength.
Novel 2-cyanoacrylic monomers have been synthesized according to the scheme 3. The esters have been obtained using modified chloroanhydride method from 2-cyanoacrylic acid prepared by high temperature vacuum pyrolisis of ethyl-2-cyanoacrylate[30].



Scheme 3. Synthesis of perfluorinated 2-cyanoacrylates

The matrix copolymer has been obtained by anionic copolymerization of the monomers listed in the table 1.

Table 1. Matrix material monomer content

Content	Mass, mg	Functions
Ethyl-2-cyanoacrylate	100	The main component of matrix material
2-Cyanoacrylic acid	5	Anionic polymerization retardant, imidizing agent
Perfluorophenyl-2-cyanoacrylate (compound A on Scheme 3)	5	Reactive comonomer, increases collagen content
1,1,1,4,4,4-hexafluoro-2,3-bis(trifluoromethyl)butane-2,3-diyl bis(2-cyanoprop-2-enoate) (compound B on Scheme 3)	5	Biodegradation rate regulator
Collagen	50	Bone morphogenetic protein analog

The use of anionic polymerization retardant is necessary to control the process. 2-cyanoacrylic acid is a multifunctional component: from one hand it is a very effective polymerization retardant, 5 mass.% content gives effective control of the process; from another hand it provides increased hydrolysis stability followed by intramolecular imidization of the main polymeric chain. The reaction occurs in mild conditions at physiological temperature close to 45°C (Fig. 2).

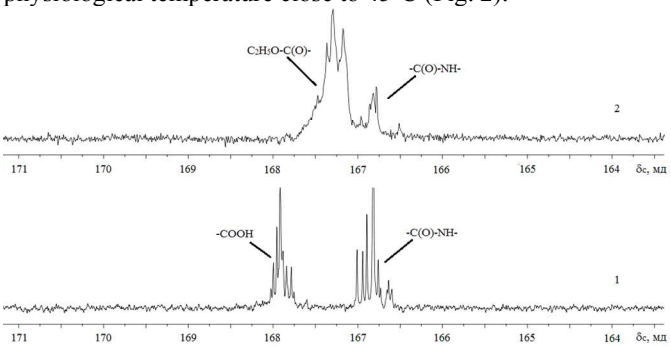


Figure 2. Fragment of ^{13}C NMR spectra of poly-2-cyanoacrylic acid (1) and 2-cyanoacrylic acid/ethyl-2-cyanoacrylate copolymer (2)

Preparation of poly-2-cyanoacrylate nanocapsules

Insulin encapsulation has been carried out using interfacial polymerization of 2-cyanoacrylic monomer in two phase polymerization media comprising immiscible aqueous solution of two water-soluble polymers: carboxymethyl cellulose (CMC) and dextran. CMC solution has been used as discrete phase attracting insulin in form of interpolymer complex. It gives distribution coefficient between discrete and continuous phase equal 70. In such a manner almost all the active was located inside droplets of CMC solution dispersed in the continuous phase of dextran. The shell of capsules comprises ethyl-2-cyanoacrylate polymerized by anionic polymerization at room temperature without any additional initiator. Actually it's a unique technology specially developed for insulin encapsulation without damage of its ternary structure and biological activity[31,32].

The capsules have average diameter around 200 nm and quite narrow size distribution (Fig. 3).

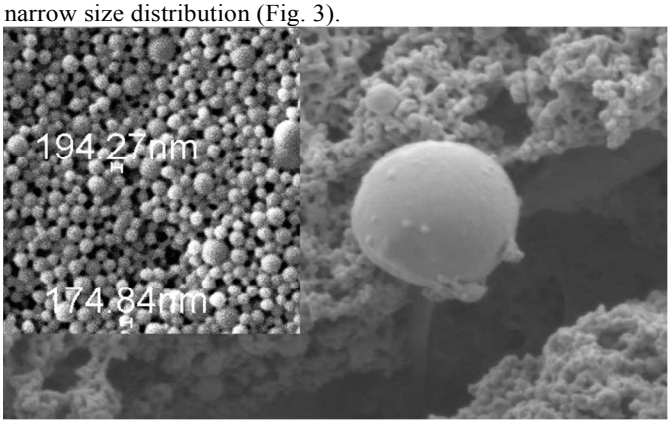
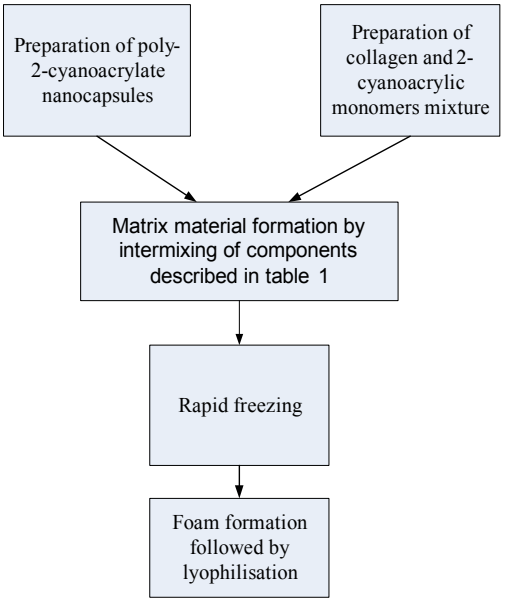


Figure 3. SEM image of poly-2-cyanoacrylate nanocapsules

Isolation of capsules comprises stage of lyophilisation followed by washing out components of polymerization medium. The freezing results partial insulin inactivation, however not less than 25% of its initial activity still remains. It is much more than enough to provide the material with wound hilling properties.

Composite foam material formation

Preparation of foam material comprises stages according to the scheme 3.



Scheme 4. Block-scheme of porous implant preparation

Initial composition contains three components: the matrix material, insulin containing nanocapsules and polymerization medium. Then it was frozen with formation of solid block giving foam after water evaporation by lyophilisation. As

intermixing results bicontinuous phase type mixture, the matrix : polymerization medium ratio defines pore size in the foam material. The pore size optimization should meet requirements on vascularization and growing cells immobilization. The actual pore size is in the range of 100 – 200 μm. Material has open-pore structure, which is optimal for vascular growth throughout the bulk of tissue substituting implant (Fig. 4).

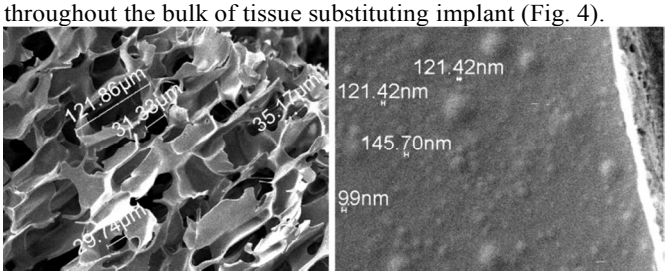


Figure 4. SEM image of porous material (left) and nanocapsules incorporated into its wall (right)

Capsules are incorporated into the wall close to its surface. They are hollow inside, that is spectacular demonstrated on the crashed wall image (Fig. 4).

In such a manner foam material contains collagen covalently bonded to the matrix and insulin filled nanocapsules incorporated into the wall.

Drug release and rate of biodegradation

Insulin and collagen release dynamics was measured by approximation of absorbance growth rate using equation:

$$A = \sum_{i=1}^{i=4} a_i \bullet t^i ,$$

where a_i – polynomial approximant coefficient, t – reaction time, days.

Differentiation of aforesaid equation resulted absorbance growth dynamics and the ratio of latter to its maximum value results protein release rate in mass. %:

$$r = \frac{1}{A_\infty}\frac{dA}{dt} ,$$

where r – insulin (collagen) release rate, mass.%/day, A_∞ - maximum value of absorbance after complete release of protein.

Insulin elution is completely controlled by diffusion through the wall of nanocapsules. The release, described by Fick's diffusion equation, is practically finished after 6 days in tests *in vitro* (Fig. 5).

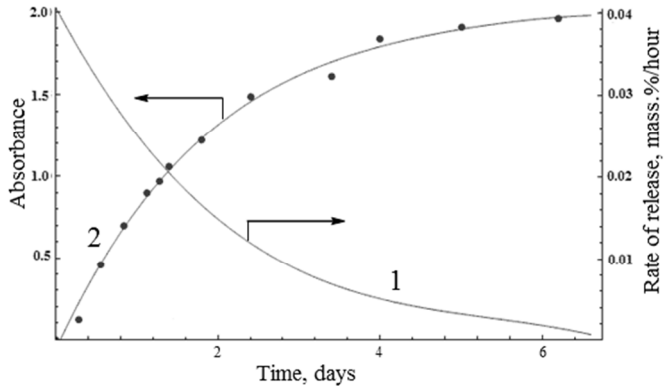


Figure 5. Insulin release dynamics

Collagen elution dynamics is much more complex, however it is typical for polymeric implants (Fig. 6).

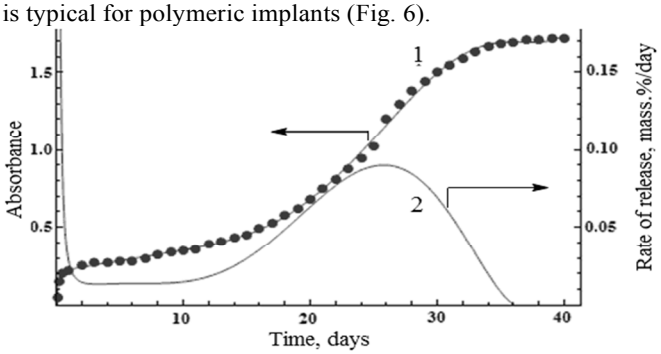


Figure 6. Collagen release dynamics

At the first stage during several days small amount of unbonded collagen is eluted. The unbonded collagen quantity is equal 0.21/1.72 mass.%. At the second stage degradation occurs on the surface without its fragmentation. It lasts for 10 days and do not result significant change on the surface area of the foam material. It can be described by zero-order equation. Fragmentation of implant and bio-erosion on the third stage results significant growth of the elution rate. This stage lasts for 25 days resulting almost complete dissolution of the biodegradable foam material.

A zero-order constant of collagen release on the second stage can be calculated by equation:

$$k = r \bullet \delta \bullet \rho \bullet \varphi ,$$

where δ – polymer wall thickness, φ – collagen content, mass.%, ρ – polymer density, g/sm^3.

Reaction rate constant is equal k = $1.18 \cdot 10^{-7}$ at 25°C and pH = 6.8 – 7.2. The effect of pH on collagen elution rate is worth emphasize.

Toxicity of foam material

Hydrolitic degradation of foam material (Scheme 1) results formaldehyde formation accompanied with non toxic ethylcyanoacetate. Although the aldehyde release was never registered its possibility must be taken into account. Peptide components provide additional safety in this case. It prevents potential formaldehyde release from the area close to degrading material by converting it into non toxic Schiff's base products even in case it is do formed.

The study carried out by P. Couvreur spectacular demonstrated that both biodegradation rate and toxicity of alkyl-2-cyanoacrylates are controlled by the size of the chain in the ester moiety[33]. The esters with the bigger chain are less toxic due to slow degradation. It's applicable not only to individual cyanoacrylic polymers like polymethyl-, polybutyl-, polyisobutyl-2-cyanoacrylate and also to copolymers and compositions.

We monitor the rate of biodegradation as the time required for 50% drop of strength on enzymatic hydrolysis of samples. It is inversely proportional to collagen – cyanoacrylate ratio and strongly depends on the content of fluorine containing cross-linking monomer (compound B on Scheme 3).

Most of the *in vitro* toxicity models provide absorption of active by the cell, however, the uptake mechanisms substantially differ for nanocapsules and for soluble products of foam material degradation. The rate of absorption of solid capsules by test culture critically depends on the rate of phagocytosis while it is not so important for soluble products. It impacts test result and requires specific cell cultures. We have

used the rat hepatosytes method to monitor both particles and soluble products toxicity proposed by P. Couvreur[33]. It has been double checked with test using isolated mouse macrophages. Scopes of dose was calculated as the number of particles per cell for nanocapsules and concentration (mg/ml) for degradation products.

Insulin is not inert filler in this case. To monitor specific toxicity of capsules it has been replaced with inactive peptide keeping formaldehyde neutralization capacity. Then samples have been tested both with and without protein inside (Table 2).

Polymer composition (%:%)	Material	Toxicity*		
		Suspensions Mouse macrophages test TC_{50}		Degradation products Rat hepatocytes tests TC_{50} (mg/ml)
		Particles per cell	mg/ml	
Polyethyl-2-cyanoacrylate	Empty Capsules	$5x10^4$	0.8	
	Bulk material			10
Polyethyl-2-cyanoacrylate: degradation inhibitors 90:10	Empty Capsules	$2x10^6$	2.8	
	Bulk material			>20
Polyethyl-2-cyanoacrylate:collagen 90:10	Protein filled capsules	$>5x10^6$	>5	
	Bulk material			>20
Polyethyl-2-cyanoacrylate:collagen: degradation inhibitors 62:31:7	Protein filled capsules	$>5x10^6$	>5	
	Bulk material			>20

*The calculation of dose has been done using number of particles per cell for nanocapsules test and scopes of concentration (mg/ml) for degradation products.

In such a manner both peptide additive and degradation retard results in toxicity decrease.

Influence of polycyanoacrylate porous scaffold on proliferation and differentiation ability of Th1 cells

The study has been done using Th1 primary cell culture isolated from third molars teeth of healthy 16 years old patient. Teeth were derived according to medical prescription. Morphological and phenotypic properties of Th1 cell culture are similar to those of mesenchymal stem cells from clonogenic properties stand point. Both have *in vitro* and *in vivo* proliferation ability and multipotent differentiation directions. Proliferation *in vitro* has been evaluated by standard alkaline phosphatase activity test. DBM has been used as a gold standard. Th1 cell proliferation and differentiation are interdependent, both characteristics have been studied. It appeared that polycyanoacrylate scaffold stimulates proliferation of Th1 cells in osteogenetic direction at 10 mg/ml concentration of its suspension. The osteogenetic differentiation character is proved by appearance of a large amount of calcificates in the area of the cell growth (Fig. 7).

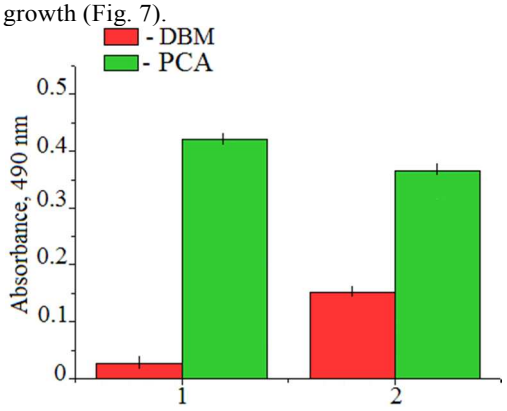


Fig. 7. Alkaline phosphatase activity (1) and calcification extent (2) of Th1 cell culture

Alkaline phosphataze expression is a marker of any multipotent mesenchimal and osteogenetic differentiated cells. The difference in AP expression character between DBM and PCA suggests the effect of the scaffold on heterogeneity of Th1 cell culture. AP activity increase is also observed in the area of calcificate formation. It demonstrates the influence of material on proliferation and differentiation of cell culture towards osteogenetic direction (Fig. 7).

***in vivo* study of poly-2-cyanoacrylate porous scaffold biological activity**

The bone regenerate formation was observed in each experiment after 30 and 45 days of implantation. Bone binding between cephalic and distal ends of resected tibia was clearly demonstrated in two experiments. One rat had a signified calcification of implanted material.

X-Ray graphical tissue density in the area of bone defect had an average value 250±25 HU in case of demineralized bone matrix (DBM). Tissue with average density of 500 HU, which is indicative for osteochondral regenerate, is absent in bone diastasis area. Average tissue density for polycyanoacrylate scaffold is 950±100 HU, that corresponds cartilaginous and bone tissue formation. However, the density is still lower than that of splenial bone of nonoperated rat (2000±200 HU).

Clinical performance of polycyanoacrylate implant rather differs from DBM. Cartilaginous tissue becomes observable after 30 and 45 days of experiment and its amount is vastly greater. The sample was also subjected to active enzyme biodegradation. Neoformed blood vessels have grown through trabeculums of implant.

The central part of implant was fulfilled with trabeculums of woven bone tissue. Osteotylus intensively appears along the major axis of tibia. Its structure is similar to that of metaphysial bone growth plate. Cartilaginous tissue is located on the surface of polycyanoacrylate implant that is associated as 3D matrix structure. A histological pattern is characteristic to bone tissue regeneration occurring by indirect endochronic osteogenesis mechanism. All of the observations together are characteristic for expressed regeneration process in implantation region (Fig. 8).

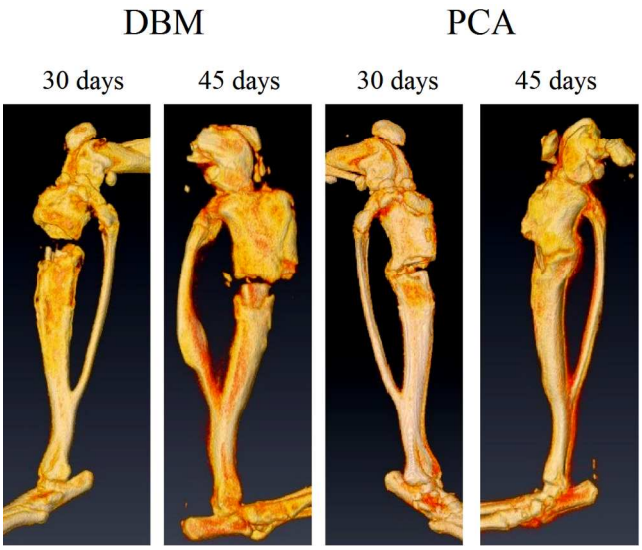


Fig. 8. X-Ray tomogram of rat tibia after 30 and 45 days of implantation

Experimental

Commercially available chemicals of appropriate purification were purchased from Sigma Aldrich and used as received.
SEM images were recorded on Nanolab 2100 (Bausch & Lomb) instrument at 25°C.
Dynamics of collagen and insulin release was measured *in vitro* by Tablet Dissolution System.
Mechanical properties of the foam material were measured as compression strength of cylinder samples (5 mm diameter) at 10% deformation.
Time of the degradation *in vitro* was measured using collagenase solution in HEPES at pH=6.8 - 7.2 as medium.
Cytotoxicity of nanoparticles was determined using mouse microphages test, while that of the product of degradation was measured by rat hepatocytes test.
2-Cyanoacrylic acid, pentafluorophenyl 2-cyanoprop-2-enoate and 1,1,1,4,4,4-hexafluoro-2,3-bis(trifluoromethyl)butane-2,3-diyl bis(2-cynoprop-2-enoate) were obtained by methods, described previously[30].

Cell proliferation and differentiation study

The cells were isolated by washing out the tooth pulp with DMEM/F12 medium containing penicillin (200 mcg/ml) and streptomycin (200 mcg/ml). The cells were precipitated by centrifugation, treated with trypsin/EDTA (0.25/0.02 mass.%) solution (30 min, 37°C), reprecipitated and cultivated in DMEM/F12 medium (1:1) in the presence of fetal bovine calf serum (FBS) and penicillin/streptomycin (100 mcg/ml) in carbon dioxide atmosphere (5 vol.%). Then the cell culture was resuspended in trypsin/EDTA (0.25/0.02 mass.%) solution and placed into 96 basins culture plate (NUNC) in amount of 50000 cells per 1 sm2. The cultivation medium was added as mentioned before and then it was twice replaced with polycyanoacrylate scaffold containing suspension (10 mg/ml in the same medium) after 18 hours and 4 days of the experiment respectively. Differentiation and proliferation was estimated after 7 days of cultivation.
Calcification extent was estimated by dyeing of calcium phosphate with Alizarin Red (pH=4.1). The cultivated cells were washed out with 0.01 M phosphate buffer (pH=7.4) and placed into formaldehyde solution (3.7 mass.%) for 20 minutes. After that, the cells were washed with distilled water followed by dyeing with Alizarin Red. The color grade of calcium compounds was estimated by measurement of absorbance at 490 nm using 680 BIO-RAD spectrophotometer. Empty basins of culture plate were used as a comparative sample.
AP activity was measured using Alkaline phosphataze kit, Sigma 86-R reagents. Cell coloration degree was estimated by measurement of absorbance at 490 nm.

In vivo study of biological activity of polycyanoacrylate porous scaffold

The experiments were carried out using 5 Wistar rats (180 gr). The study has been done in N. Priorov Central Institute of Traumatology and Orthopedics of Russian Ministry of Health according to the Guide for the care and use of laboratory animals, eighth edition (National research council), directive 2010/63/EU and in accordance with decision of ethical committee of the Institution.
Partial resection of rat tibia was performed in aseptic conditions after ketamine narcosis. The tibia diastasis was formed by extraction of 5 mm bone segment followed by implantation of test sample. Cephalic and distal ends of tibia resected portion were stabilized by carbon nail fixated along medullary canal. The operation was performed on left and right legs simultaneously.
Demineralized bone matrix (DBM) was used for control observation of experiment. Osteosynthesis was carried out on the left hindpaw of rat by implantation of xenogenic DBM treated with guanidine hydrochloride (1 mass.%). Polycyanoacrylate sample was implanted in the same way to the right hindpaw of rat. The implantation period lasted 60 days.
Osteoconductivity of polycyanoacrylate porous material was estimated by regenerative process observation at bone resection area using X-Ray tomographic densimetry and X-Ray tomography study after 30 and 60 days of implantation.
X-Ray tomographic densimetry was used for estimation of osteohondrous regenerate density using SkyScan 1176 microtomograph. Density distribution was represented on diagram described in Hounsfield units for DBM and polycyanoacrylate samples as well.
X-Ray tomography of autopsy material was carried out after 30, 45 and 60 days after implantation using ScyScan 1176 microtomograph. 3D models were build using Avisio 7 software.

Synthesis of poly-2-cyanoacrylate nanocapsules

10 g of dextran and 1.14 g of PEG were dissolved in 50 ml of water by mixing and heating to 80°C. After cooling, the mixture was allowed to stand and the layers formed separated in a separatory funnel. 0.2 ml of the upper phase (primarily PEG) was added to 20 ml of the lower phase (primarily dextran) along with 50 mg of insulin. The mixture was next placed in a cooled sonication vessel for 2 min with continuous cooling. 0.5 ml of ethyl-2-cyanoacrylate was then added and sonication with continuous cooling in an ice bath continued for a further 10 min. After this time the suspension was transferred to a magnetic stirrer and stirred for 8 hours. Obtained nanocapsules were precipitated by centrifugation and the upper layer was decanted.

Preparation of poly-2-cyanoacrylate porous material

To a cooled concentrated suspension of poly-2-cyanoacrylate nanocapsules was added solution of 50 mg of dermal collagen in water and stirred for 10 min. Then the mixture, comprising, 0.025 g of 2-cyanoacrylic acid, 0.025 g of pentafluorophenyl 2-cyanoprop-2-enoate, 0.025 g of 1,1,1,4,4,4-hexafluoro-2,3-bis(trifluoromethyl)butane-2,3-diyl bis(2-cynoprop-2-enoate) and 0.5 ml of ethyl-2-cyanoacrylate was added dropwise to a suspension of nanocapsules and stirred for 1 hour. Further the mixture was rapidly cooled with liquid nitrogen and lyophilized during 3 days resulting a porous poly-2-cyanoacrylate material containing poly-2-cyanoacrylate nanocapsules.

Conclusions

It has been done the proof of concept study that demonstrated the efficacy of approach for design of biodegradable bone substituting foam material with controlled rate of biodegradation able to elute two actives with independent (bimodal) release modes. The material doesn't initiate abscess formation and is able to conduct bone tissue growth.

Notes and references

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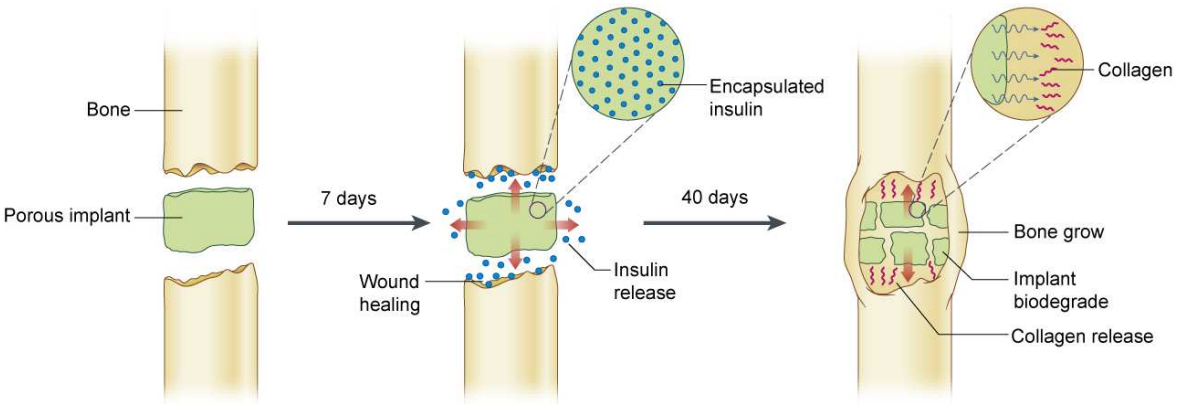

It has been developed a novel polycyanoacrylate based bone substituting foam material providing independent release of two polypeptide actives with followed biodegradation and bone substitution.