Collagen-based Composite and Hybrid Bone Implant Materials: Structure and Property Characterization

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***Abstract*— Modern bone implants and their scaffolds are evolving to become ever more complex engineering objects that possess the features of hierarchically structured composites and hybrid materials. Collagen, hydroxyapatite (HAp) and ultra-high-molecular-weight polyethylene (UHMWPE) are biocompatible materials with well-proven track record of use in bone tissue engineering. They can be purposefully combined and structured in order to fabricate engineered biomaterials. We present some recent achievements in the fabrication of cellular scaffolds from Collagen-HAp and Collagen-UHMWPE by freeze casting and drying. The samples were characterized using Raman spectroscopy, FTIR, SEM, contact angle measurements, and biocompatibility tests.**

***Index Terms*—Bone graft, biocompatibility, contact angle, collagen, hydroxyapatite, FTIR spectroscopy, freeze-casting, Raman spectroscopy, SEM, tissue scaffold, vibrational spectroscopy, UHMWPE.**

I. INTRODUCTION

Reconstructive surgery applications call for continuous improvement of materials for implants and prosthetic materials. A wide range of engineered materials for bone replacement have been proposed and developed with the aim of enhancing the mechanical performance, bioinertness or biodegradability to achieve the best functional match to the natural properties. There are rigorous requirements to the fabrication of implantable medical materials in terms of sterility, biological compatibility, and longer term effects on human health.

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A wide range of HAp-based biomaterials has been put forward, ranging from bioglass to ceramic composites. Since hydroxyapatite (HAp) is the principal mineral constitutent of mammalian bone, and hence forms a natural candidate material for bone prosthesis. Its presence accelerates bone regeneration and osseointegration into a living body.

Hydroxyapatite is widely used as the main component of bioactive materials characterized by a high biocompatibility index. However, in the absence of tight integration with organic binder such as collagen, bulk HAp possesses rather limited strength. Therefore, the formulation and use of multi-component composite materials based on HAp appears to be a promising avenue for further exploration. At the first stages following implantation, resorption takes place during which exogenous HAp is dissolved, and its local concentration is decreased. Subsequent osseointegration involves the re-deposition of biogenic HAp, resulting in the formation of natural bone tissue replacement.

Another crucial component of mammalian bone (and other
tissues, including skin) is collagen, a protein fibre with complex hierarchical structure. There are extensive reports of the use of collagen for fabricating scaffolds and artificial tissue replacement grafts. According to the literature, scaffolds were obtained by freeze-casting to create aligned open porosity, followed by lyophilization and crosslinking to improve the mechanical performance for further osteintegration of bone graft [1,2].

Structural and functional changes during processing may affect biocompatbility and impede integration. Therefore, suitable characterization needs to be carried out to monitor the consequences of processing, ageing and regeneration, e.g. by Raman spectroscopy, FTIR, SEM, etc. [3].

UHMWPE is another popular prosthetic material that is highly bioinert, i.e. hardly interacts with the host tissue(s). A hybrid material derived from UHMWPE impregnated with collagen gel may offer interesting opportunities for reconstructive surgery. In comparison with brittle HAp, UHMWPE shows good strength and wear resistance required for use in artificial joints.

In the present paper, we report experimental results on the structure and properties of collagen-based composites and hybrids obtained with a number of manufacturing techniques including freeze casting/drying of collagen-HAp slurries, hot molding and collagen impregnation of bioinert polymers to obtain the combination of biocompatibility and high mechanical performance. Raman spectroscopy [3] and

ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

other methods of characterization used herein allow comprehensive understanding of the structure-property relationship in the class of biomaterials studied.

II. Materials and Methods

A. Collagen/HAp Sample Preparation

2 wt.% collagen solution in acetic acid (BioProduct Ltd., Russia) was mixed with HAp powder (NGO Polystom, Moscow) to obtain a slurry with the mass ratio 70:30 of collagen to HAp, respectively. The slurry was stirred using alumina milling bodies (d = 5mm) using SPEX™ SamplePrep8000M Mixer/Mill (Stanmore, UK) for 20 min at the 1425 rpm. A special cylindrical mold was designed consisting of PLA 3D-printed walls and thermoconductive aluminium base to generate predominant vertical heat flow and temperature gradient for guided formation of ice crystals during freezing. The slurry was installed into cylindrical molds, and cooled at the bottom by placing the molds into liquid nitrogen. Frozen slurries were freeze dried in FreeZone 12 Liter -84C Console Freeze Dryer with Stoppering Tray Dryer (Labconco®, USA) at -30 °C for 48 h.

B. Preparation of References for Spectroscopy

The thin collagen films as a reference samples for FTIR and Raman spectroscopy investigations were prepared by drying the collagen gel (2 wt.% collagen solution in acetic acid supplied by BioProduct Ltd., Russia) at ambient temperature for 2 days.

Deimmunized humerus bone (referred to as DMB hereafter) taken from ethically mortified dog was provided by N.N. Blokhin MRCO Institute (Moscow, Russia) in accordance with the regulations in force [4].

C. Porous UHMWPE sample preparation

UHMWPE powder (GUR 4120, Ticona, molecular weight of $5 \cdot 10^6$ g/mol) was used for the porous sample fabrication. NaCl was used as the soluble filler with the following granulometric parameters: up to 0.8 mm – 75 %; 0.8 to 1.2 mm – 25 %.

Solid-state mixing of UHMWPE powder and NaCl was performed in a planetary ball mill Fritsch Pulverisette 5 (Fritsch GmbH, Germany). The powders were placed in 500 ml agate bowls with corundum grinding balls 10 mm in diameter used as milling bodies. The rotation speed was 90 rpm. Mixing was carried out under low energy conditions to prevent the reduction of salt particle size. As a result, a mixture of UHMWPE and NaCl of specific composition was obtained, with the mass ratio 1:9 [5].

Highly porous UHMWPE scaffolds were produced by the hot molding under the pressure of 50 MPa and temperature of 180 °C for 2 hours. Subsequently, the obtained specimens were rinsed with distilled water to remove the salt. The samples were dried at 70 °C for 1 hour to remove adsorbed water. The obtained samples were cylinders with the diameter of 26 mm and height of 10 mm.

D. UHMWPE/collagen hybrid preparation

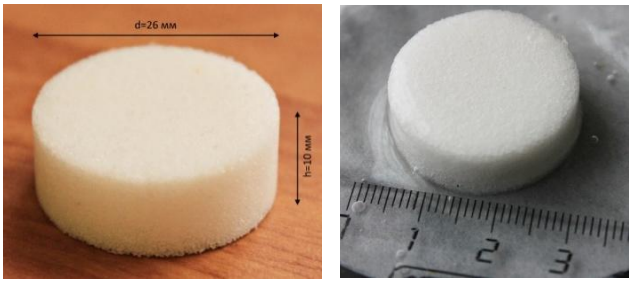
Porous UHMWPE was impregnated with 2 wt. % collagen solution using three different routes.

The first route comrised spreading collagen on the surface of UHMWPE as a dense layer after freezing.

The second route involved infusion through vacuum infiltration whereas air evacuated from the pores was replaced with collagen intrusion into the pores.

The third route for impregnation involved placing UHMWPE sponge into a syringe, adding collagen solution and pressing the solution through the sponge using the piston.

Afterwards, samples were freeze-dried in vacuum at −30°C for 48 h (Genesis Pilot Lyophilizer, USA). Porous samples before and after impregnation with collagen are shown in Fig. 1.



a) *b)*

Fig.1. Porous UHMWPE: a) without collagen; b) with collagen

E. Chemical Modification of UHMWPE Samples

UHMWPE is a hydrophobic polymer [6], while collagen is highly hydrophilic [7]. Thus, surface modification of UHMWPE is necessary to reduce its hydrophobicity and to achieve a satisfactory bond with collagen. Chemical modification of UHMWPE surface with chromic solution is a treatment that may be used to improve the interaction between UHMWPE and other polymers due to its efficiency, low cost and simplicity [8,9]. All samples were first cleaned with ethanol and distilled water. This was followed by air drying at room temperature prior to chemical modification by the mixture of potassium dichromate $K_2Cr_2O_7$, sulfuric acid H_2SO_4, and water. UHMWPE samples were immersed in the chromic solution for about 30 min at room temperature, and then rinsed with distilled water 10 times. The modified samples were dried in a laboratory fume hood at room temperature for 12 hours.

F. Contact Angle Measurement

Contact angle was measured with EasyDrop Shape Analyzer (KRÜSS, Germany) using static sessile drop method at room temperature. For every measurement, 5 μL of water and collagen solution were dropped on the sample surface by the dosing dispenser. The baseline and contact angle were determined via Drop Shape Analysis.

G. FTIR Spectroscopy

Attenuated Total Reflectance Fourier transform infrared spectroscopy (ATR FTIR) was used to examine the chemical structure of collagen derived biomaterials. It is an established method for study of the structural features of organic and inorganic compounds [10]. Attenuated Total

ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

Reflectance (ATR) FTIR spectra of collagen based hybrids were collected in the wavelength range of 4000–600 cm^{-1} using a stand-alone FT-IR microscope LUMOS (Bruker) equipped with a Ge ATR crystal. Analogous spectra of collagen film and Hap powder were obtained as references.

ATR FTIR spectra of the porous UHMWPE samples before and after surface modification and spectra of the hybrid UHMWPE/collagen were recorded in the wavelength range of 4000–400 cm^{-1} using Nicolet 380 (Thermo Scientific, USA) spectrometer equipped with diamond ATR crystall.

H. Raman Spectroscopy

Raman spectroscopy was applied to trace the collagen evolution with reference samples - deimmunized bone matrix (DMB) and dried collagen film. The samples were irradiated with laser source with wavelength 532 nm in the DXR™2xi Raman imaging microscope (Thermo Fisher Scientific, USA).

The spectra of UHMWPE-collagen hybrid were compared with pure UHMWPE to characterize the presented collagen.

I. Scanning Electron Microscopy

The morphology of porous and hybrid scaffolds was studied with Scanning Electron Microscopy (SEM). SEM was performed with the help of VEGA3 microscope (Tescan, Brno, Czech Republic) with the accelerating voltage of 5 kV and 20 kV and analyzing software VEGA TC. For sample preparation the surface of samples was platinum coated by Auto Fine Coater JFC-1600 (JEOL, USA). Porosity of the UHMWPE sample was calculated using the equation (1)

$$P = \left(1 - \frac{V_{por}}{V}\right) \times 100\ \% \quad (1)$$

where V_{por} is a measured volume of a porous sample, V is a calculated volume of a non-porous sample of the same size. A volume of a porous sample was calculated by multiplying length, width and thickness of standard porous sample, thus the average V_{por} value was calculated.

J. In vitro Biocompatibility Tests

The high hemocompatibility is vital to pursue the development of biomaterials of A and B categories in accordance with ISO 10993.

The UHMWPE-collagen hybrids were washed with 40 ml of sterile 0.9% sodium chloride solution (PanEco, Russia) prior to analysis. Two sterile samples were evaluated with the following hemocompatibility criteria: cytotoxicity and induced hemolysis. Each sample was divided into 5 parts, with weight 0.020 ± 0.003 g.

For the required test, the erythrocytes were presented after the repeating of centrifugation for two times. Erythrocytes were isolated from the peripheral blood of a healthy donor stabilized with heparin sodium (Binergia CJSC, Russia) - 200 IU / ml. The blood was diluted with the mass ratio 1:10 of blood to the sterile 0.9% NaCl solution and centrifuged at 3000 rpm at 20° C for 2 min, followed by removal of the supernatant. Hence, a suspension of erythrocytes was obtained by adding 6.5 ml of 0.9 wt.% NaCl solution to the washed erythrocytes.

Mononuclear leukocytes (ML) were yield by separating the blood of two healthy donors on a Ficoll gradient with the density 1.077 g/ml, followed by a wash with 0.9 wt.% NaCl solution. A working assay of leukocytes was obtained by adding to the sediment containing ML, 6.5 ml of DMEM medium with 10% fetal calf serum, penicillin-ampicillin, glutamine. The concentration of ML in the working suspension reached up to 5.4×10^4 cells per ml, the viability was 94%.

a Induced Hemolysis (IG) Assay

The erythrocyte suspension was poured into 0.5 ml wells of a 48-well plate (NUNC). Samples were incubated with a suspension of erythrocytes at 37 ° C for 4 hours. The control was a suspension of red blood cells, incubated under the same conditions without the addition of samples. The dynamics of hemolysis was evaluated at 1, 2 and 4 hours after the start of incubation. Thus, the optical density (OD) of the supernatant was measured at 540 nm using Labsystems MS Multiscan plate reader to determine the concentration of hemoglobin. Samples were evaluated as hemocompatible if the IG value did not exceed 10% at 4 h after the start of co-incubation of the cells with the samples [11].

b Cytotoxicity Assessment

Suspension of ML of each donor was poured into 1 ml of the wells of a 48-well plate (NUNC). Samples were incubated with cells for 24 hours at 37 C and 5 % CO_2 environment. Control was a suspension of ML incubated in the same conditions without adding samples. The study was performed using the MTT test to assess the metabolic activity of cells, based on the ability of NADPH-dependent cellular oxidoreductase enzymes to restore the yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) in insoluble formazan, which was initially a purple.

The result was evaluated by measuring the OD of a formazan solution, which was directly proportional to the level of metabolic activity of the cells in the well, using an MS Multiscan tablet reader. The cytotoxicity of the material was evaluated in the ratio of OD in the wells with samples to the intact control of ML, expressed as a percentage. Samples were assessed as hemocompatible if the OD of formazan solution in the sample wells was higher or did not significantly differ from the OD of the intact control ($p > 0.05$) [12].

c Statistics

All experiments were performed with triplicate assays and repeated three times using separate experiments. OD measurements were presented as mean value ± standard deviation (SD). A comparative analysis of IG was performed using the t-test, and the cytotoxicity analysis was performed using the Mann – Whitney U test. The probability of repeatable results was assigned at p-values 0.05.

III. Results and Discussion

A. Contact Angle Measurement

It was established that UHMWPE porous samples before modification were hydrophobic since the contact angles were higher than 90°. The contact angle of the as-received

ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

UHMWPE sample after dropping water on the surface was 112.2° and decreases to 99.7 ° after etching by a chromic solution.

Conversely, the contact angle of UHMWPE-collagen surface after dropping collagen was 104.6° then decreased to 81.3°, as shown in Fig.2.

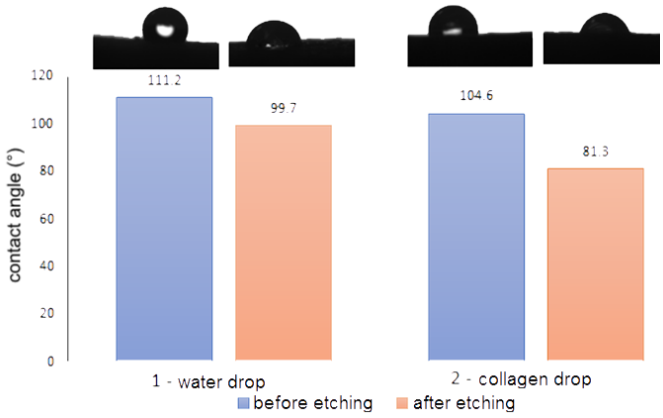


Fig. 2. Contact angle measurement of UHMWPE sample before/after modification

According to the results, the wettability of UHMWPE hybrids was improved after collagen modification. The change of contact angles can be explained by the formation of hydrophilic groups, such as hydroxyl (–OH) and carbonyl groups (–C–O, –C=O), which were identified by FTIR spectroscopy of modified samples.

B. FTIR Spectroscopy

The FTIR spectrum of collagen-Hap hybrid is presented in Fig. 3.a altogether with the spectra of pure collagen and Hap. The spectrum of pure collagen was determined from the characterization of dried collagen film. It exhibits typical collagen bands [13], namely Amide I (1638-1646 cm^{-1}), Amide II (1541-1157 cm^{-1}), Amide III (1350-1200 cm^{-1}) as well as Amide A (3312 cm^{-1}) and Amide B (2932 and 3060 cm^{-1}). The Amide A absorption corresponds to NH stretching vibration, while asymmetrical stretching vibrations of CH_2 group is shown by the Amide B band. The Amide I band is due to CO stretching vibration in polypeptide backbone. The Amide II and Amide III bands are generally associated with both the NH in-plane bending and the CN stretching vibrations [13]. The Amide III band are highly sensitive to the secondary structure folding [14]. Comparing the spectra of Collagen-Hap hybrid and neat collagen film, one may notice that they are almost identical except for differences in the Amide III band. This observation evidences that the secondary structure of collagen may be sufficiently changed or even partially destructed upon freeze-drying procedure used for the preparation of collagen-Hap hybrid. It is noted that strong band centred at 1029 cm^{-1} accompanied with weak satellites at 1088 cm^{-1} and 600 cm^{-1} observed in the spectrum of Collagen-Hap hybrid corresponds to the phosphate group vibrations of Hap verified by comparison with the reference spectrum of pure Hap powder in Fig.3.d. The bands of phosphate group located at 600, 1029, 1088 cm^{-1} and carbonate groups 876 and 1460 cm^{-1} characterize the Hap presence after freeze-casting of samples [15].

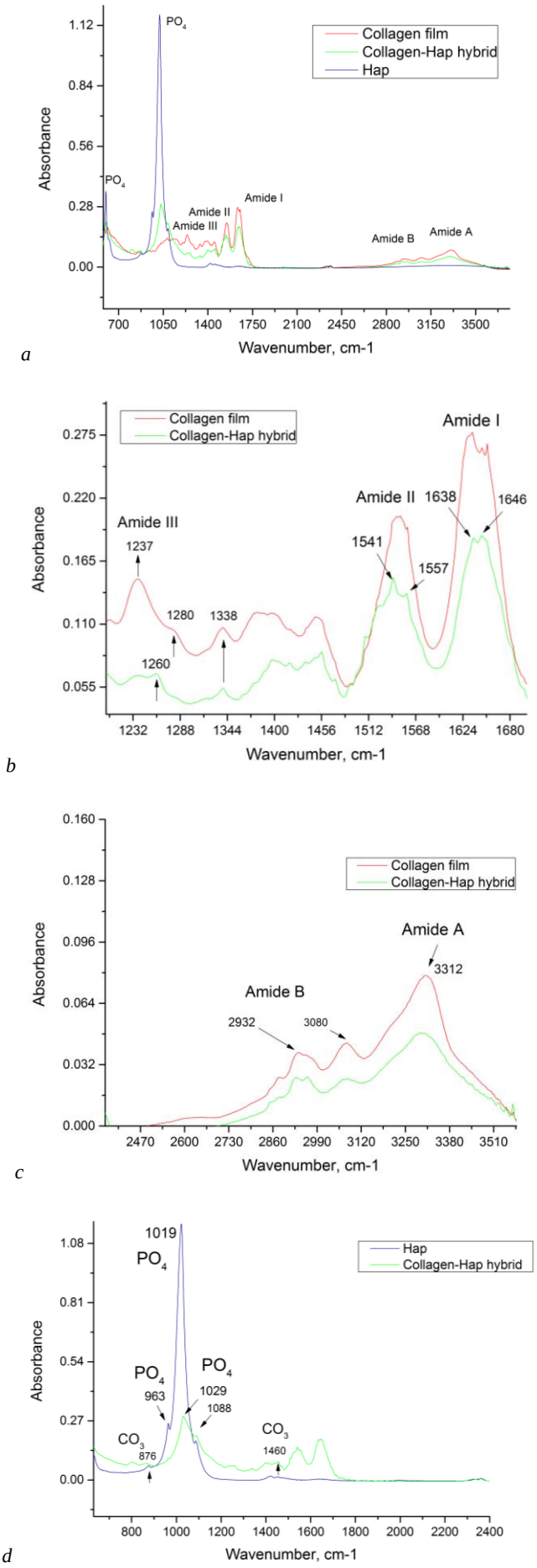


Fig. 3 FTIR spectra of dried Collagen film and hybrid with 30 wt. % of Hap

ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

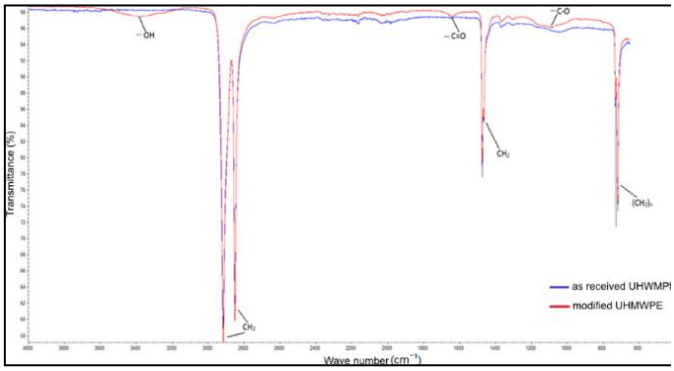


Fig.4 FTIR spectra of UHMWPE samples

UHMWPE samples were characterized by FTIR spectroscopy. Fig. 4 illustrates the FTIR spectra of as-received and chromic acid modified UHMWPE. The absorption peaks of 2914, 2847, 1471, and 717 cm^{-1} are attributed to methylene $–CH_2$ nonsymmetry stretch vibration,$–CH_2$ symmetry stretch vibration, $–CH_2$ non-symmetry changing angle vibration, and $–(CH_2)_n$ swing in plane vibration, respectively [14, 16]. A broad band around 3400 cm^{-1} observed in spectra of UHMWPE hybrid indicates the presence of hydroxyl group -OH. The vibrations including the other bands around 1633 cm^{-1} and 1120 cm^{-1} refer to oxygen based carbonyl groups –C=O and –C–O, respectively [17]. The surface etching with chromic solution causes an increase of oxygen containing group.

FTIR spectroscopy of hybrid UHMWPE/collagen surface is shown in Fig.5. The spectrum contains Amide A band located at 3304 cm^{-1}, Amide B band at 2917 cm^{-1}, Amide I band at 1653 cm^{-1}, Amide II band at 1544 cm^{-1} and Amide III band at 1237 cm^{-1}. These absorptions correspond to specific functional groups characteristic for collagen.

Hence, FTIR spectroscopy proved the presence of collagen on UHMWPE surface.

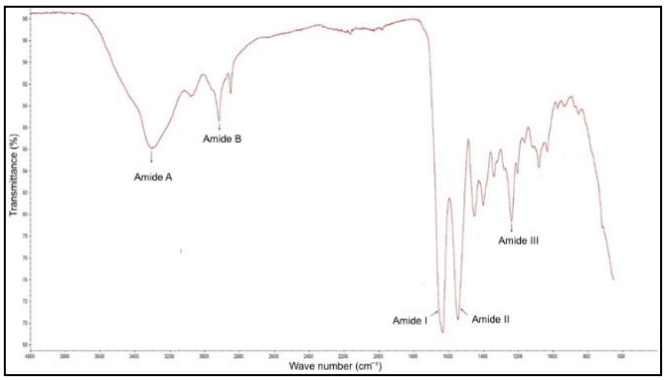


Fig.5. FTIR spectra of UHMWPE/collagen surface

FTIR spectra of a cross-section of UHMWPE/collagen hybrid and collagen film are shown in the Fig.6. A blue spectrum corresponds to collagen and a red one indicates the UHMWPE hybrid compared to the spectrum of as received UHMWPE in Fig. 4. The difference with additional peaks can be reliably attributed to collagen [14,16]. Therefore, FTIR spectra evidenced the residence of collagen in the pores of the UHMWPE sample.

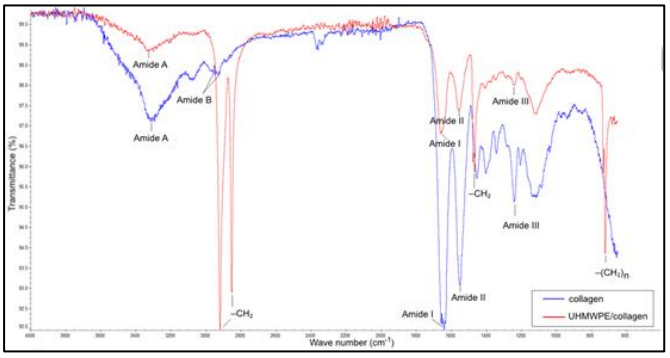


Fig.6. FTIR spectra of UHMWPE/collagen cross-section

C. Raman Spectroscopy

Raman spectrum highlights the peculiarities of different collagen/HAp composite. Due to the fact that collagen is an intricate protein that contains amino acids, the organic subregion of spectrum is manifested in the range 1200-3000 cm^{-1}. Additionally, the mineral subregion starts just short of 500 to 1200 cm^{-1} with the most intense and characteristic bands of the mineral components.

Fig.7.a demonstrates that deimmunized bone (DBM) contains massive collagen with relatively low HAp content and HAp/Collagen freeze cast/dried composite have noticeably different Raman spectra. We observe distinct peaks of HAp and weak traces of collagen gel in acetic acid solution in the spectrum. The main fingerprint of HAp is indicative for phosphate groups located in the 960 and 1045 cm^{-1}[18] for deimmunized bone matrix and collagen composite.

The Raman spectra showed similar structural compositions for the deimmunized bone matrix and freeze-dried hybrids. Fig.7. b illustrated the presence of the phosphate band occurred in 960, 1045 cm^{-1} and carbonate one occupied at 1075 cm^{-1}. The presence of HAp was evidenced by O-P-O bonds located around 588 and 597 cm^{-1} [19]. The pore size of the collagen hybrid reached up to 300 μm, thus, it was an obstacle to find the area of observation. The measurement of the DMB lets to obtain the data corresponding to the collagen with a residual amount of HAp.

Fig.7.c shows the CH_3 vibration bonds and Amide I located in the 2933 cm^{-1} and 1660 cm^{-1}, respectively[3]. The notable peak of CH_2 occurred in 1455 cm^{-1} refers to the collagen [20]. The Amide III band as a component of collagen was detected in the 1250 cm^{-1} in Fig.7.a.

The characteristic peaks of phosphate groups were evidenced in Fig. 8 after the shift of laser source from 532 nm to 780 nm. The spectrum of the collagen-Hap hybrid was performed in the range 200-3000 cm^{-1}, where the band of organics was not detected for the laser mode 780 nm. Although, the phosphate groups were indicated clearly in the spectrum. Thus, the presence of collagen was not investigated in the source with a wavelength of 780 nm due to the high amplitude of vibrations occurred after 2000 cm^{-1}.

ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

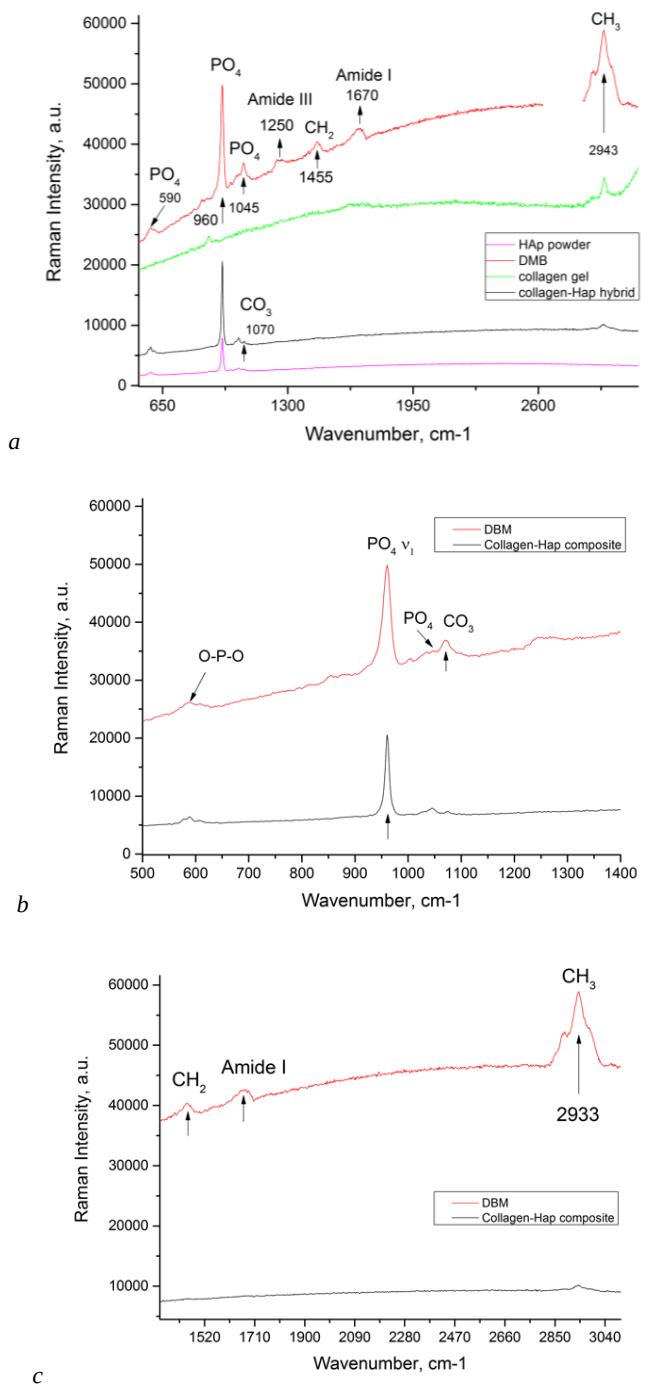


Fig.7. Raman spectra for DBM and hybrid with 30 wt. % of Hap

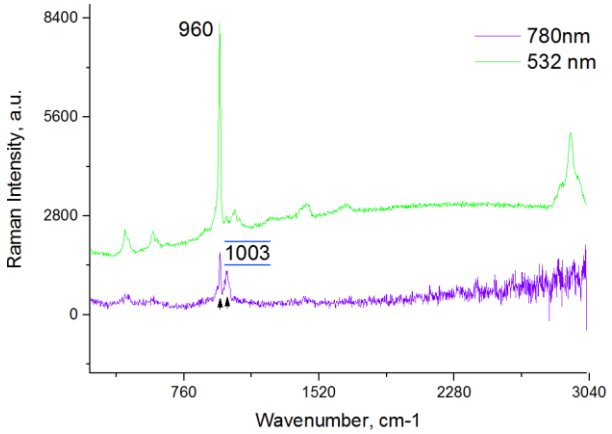


Fig.8 Raman spectra for hybrid with 30 wt. % of Hap

The specific band of UHMWPE with intensive peaks begins from 1020-1500 cm^{-1}. Fig.9.a represents polyethylene features at 1060, 1127, 1170, 1293, 1370, 1416, 1438, and 1455 cm^{-1}, with the corresponding vibrational assignments in Fig. 9.b. For the spectrum of UHMWPE the strong peaks are located at 1060 and 1127 cm^{-1} for C-C bonds and the band at 1293 cm^{-1} corresponds to the C-H bonds [21].

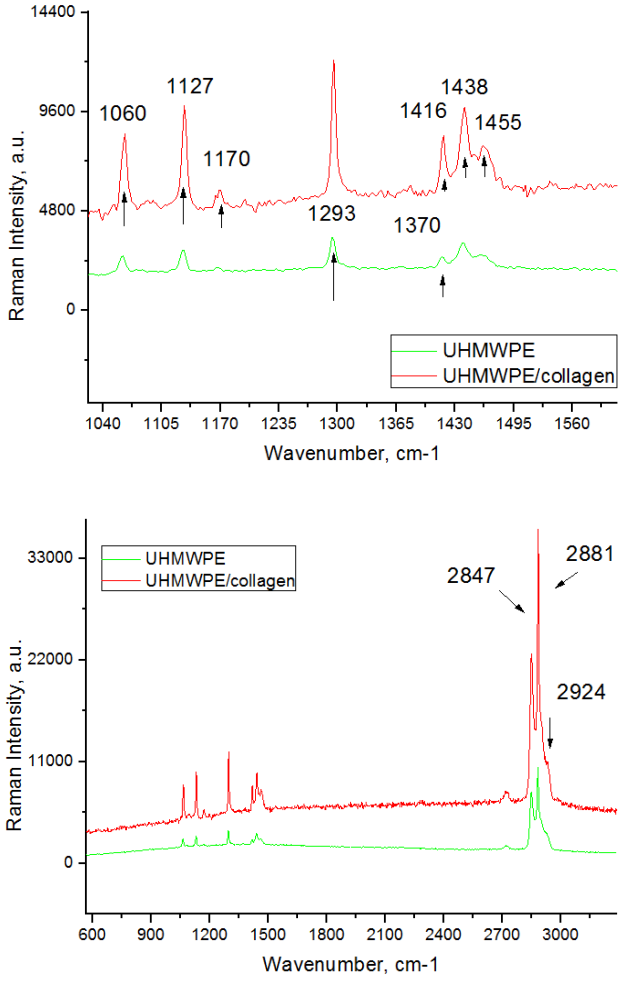


Fig.9 Raman spectra for the UHMWPE hybrids

The intensive peaks represented at 2847 and 2881 cm^{-1} indicate the presence of CH_2 symmetric stretching and CH_2 asymmetric stretching bonds, relatively. CH vibration is corresponding to the 2924 cm^{-1} at the Fig.9.b [3].

The difference between UHMWPE and UHMWPE/collagen hybrid was illustrated by broad peaks with low intensity. The spectra indicated the preserved collagen after freeze-casting and freeze-drying process. Thus, the observation using Raman spectroscopy was not carried out to observe the specific components that collagen comprises. FTIR spectrometry has greater benefits for further characterization and monitoring of collagen-based biomaterials.

ISBN: 978-988-14048-6-2
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D. SEM

SEM images of the porous UHMWPE sample showed a highly porous structure with irregular pores with size up to 315 μm. Calculated volume porosity of the sample varied from 70 to 80% (Fig.9.a). The material has open porosity and interconnected structure. The observed pore size and micro-morphology of the pores can promote cell attachment [22].

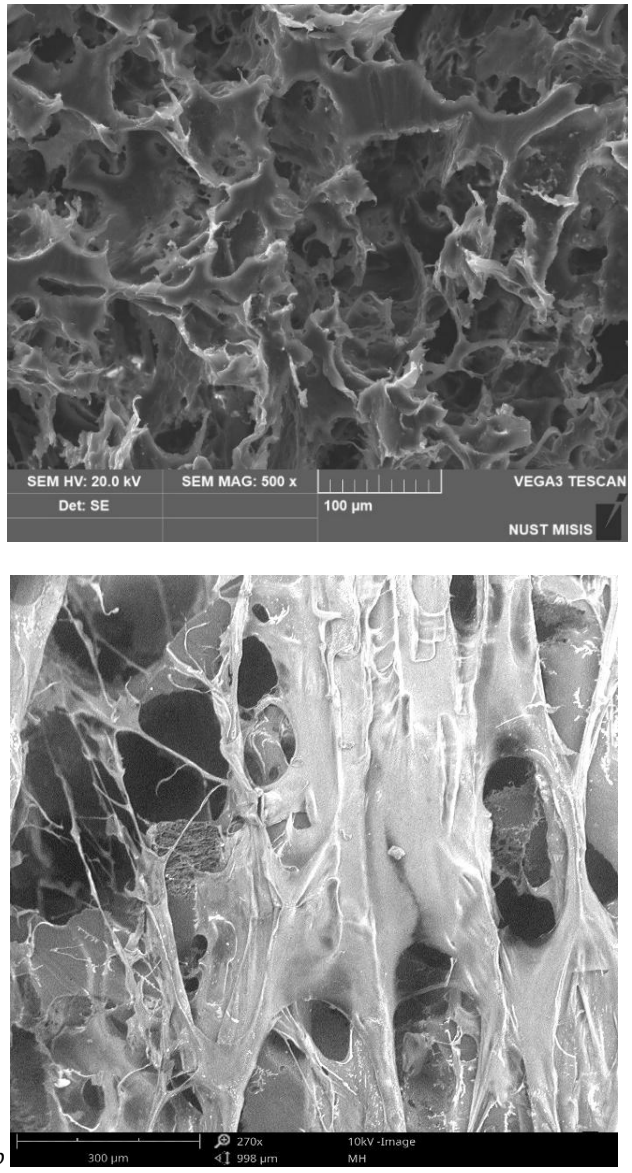

Fig. 10. SEM images of samples: a) porous UHMWPE , b) UHMWPE/collagen cross-section

SEM of the UHMWPE/collagen hybrid showed porous collagen structure within open-cell porous UHMWPE and evidenced existence of collagen inside the pores (Fig.10. b).

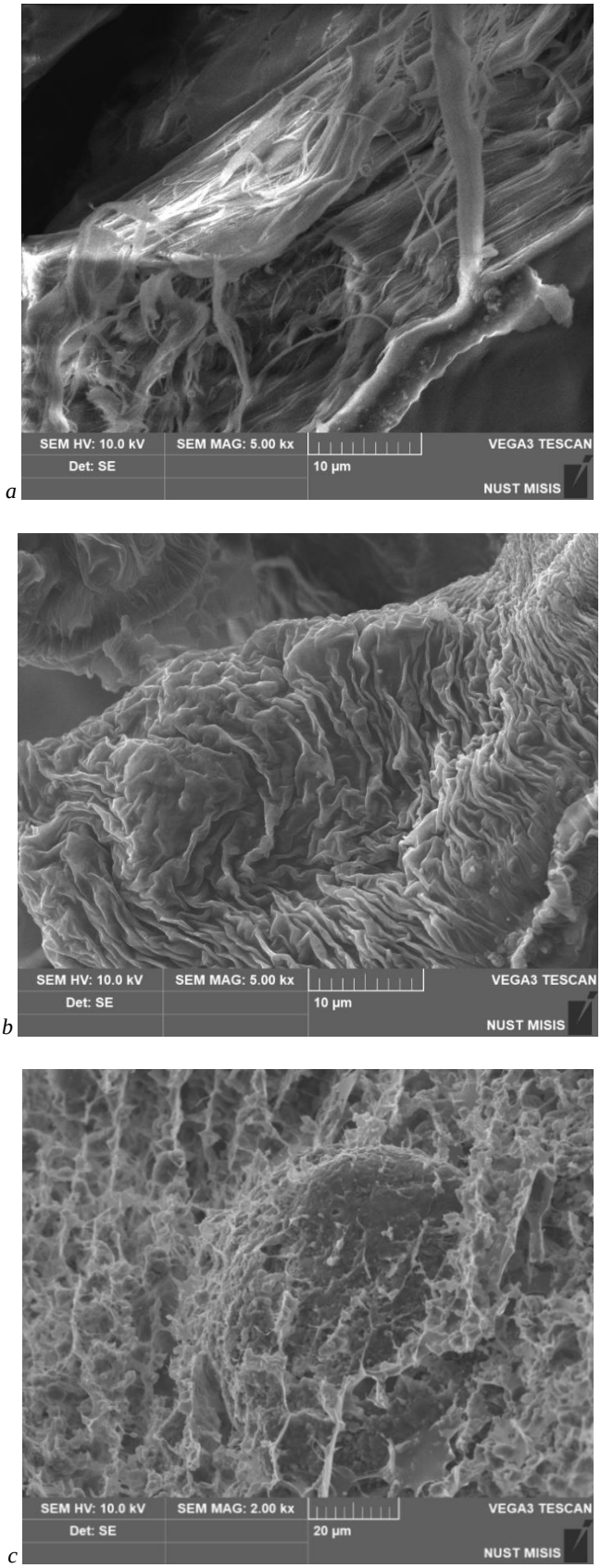

Fig.11. SEM images of a) collagen film; b) collagen freeze dried; c) collagen-HAp hybrid

ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)

Collagen fibres of dried film were presented in Fig.11.a, whereas the thickness of one fibre reached up to 2 μm.

Fig.11.b illustrated the collagen sponge obtained after freeze-drying. The distribution of fibres was modified as the hierarchically organized structure.

The morphology of collagen-Hap hybrid showed the homogeneously organized network of struts with the pore size varied from 6 to 10 μm. The microscale irregularities indicate the Hap particles embedded in the collagen cellular network in Fig. 11.c.

E. In vitro Tests

Hemolysis is referred to the rupture of red blood cells (erythrocytes) and the release of their contents into the blood plasma. To assess the impact of hybrid materials on erythrocytes a hemolysis test was performed by spectrophotometric measurement of hemoglobin release. The results demonstrated satisfactory hemocompatibility since the individual IG values were 6,0 ± 1,5 % at 4 h after the start of the experiment.

To assess the effect of samples on the metabolic activity of human blood leukocytes, the OD value of dissolved formazan was measured in the wells with the samples and in the control with intact cells after 24 h from the start of co-incubation. The change in the viability index (Viability,%) of the cells under the influence of co-incubation with the samples was calculated. The ratio of the median OD in the wells with the samples to the control for each donor and the average value was determined. It was demonstrated that samples did not negatively affect on the cell for 24 h. Their viability was 105±8%, p=0,072. Therefore, the obtained results demonstrate good hemocompatibility of hybrid samples during short-term co-incubation with cells.

IV. Conclusion

The results presented here provide a systematic characterization of hybrid composite materials for biomedical applications obtained via different processing routes. Freeze casting and drying of collagen/HAp sponges allowed the obtention of hierarchically structured porous materials. These complex poroids possess promising mechanical, chemical and biological properties, but they need to be further densified and examined in order to pave the way for their future use as bulk biodegradable bone grafts. It is anticipated that collagen-derived HAp-containing scaffolds would lead to improved resorption and promote the formation of natural bone replacement tissue.

Modification of UHMWPE through hybridization with collagen is another promising approach to improve UHMWPE-based biomaterials in terms of faster and safer body integration, for which clinical evidence needs to be sought.

Molecular spectroscopy characterization allows the identification of specific molecular bonds comprised within test samples. In the context of the present study, it suggested efficient monitoring of the preservation during processing of key elements of the chemical structure of biologically derived components such as collagen.

Acknowledgement

The project was conducted with the support of the Royal Society, UK (IEC/R2/170223), EPSRC UK (EP/P005381/1), and Zelenaya Dubrava company, Russia. We are grateful for the biocompatability tests provided by Dr. N.Yu. Anisimova, senior research scientist of N.N. Blokhin MRCO Institute (Moscow, Russia).

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ISBN: 978-988-14048-6-2
ISSN: 2078-0958 (Print); ISSN: 2078-0966 (Online)