SYNTHETIC EXTRACELLULAR MATRIX AS A SUBSTRATE FOR REGENERATIVE MEDICINE

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Abstract

The work presents materials characteristics of fibrous polysaccharide substrates (calcium alginate, CA) modified with short peptides. Three types of synthesized peptides (hexapeptides) were composed of: cysteine (C) and tryptophan (W) named - $(WWC)_2$ or cysteine (C) and tyrosine (Y) named $(YYC)_2$ or phenyloalanine (F) named 6F. The peptides size distribution (DLS method) showed that they agglomerated in an alcohol medium. These results were used to select a modification method of the fibrous substrates i.e. the peptides were deposited on the fibrous alginate substrate by the electrospraying technique. Using this method three kinds of polysaccharide- peptides systems were obtained i.e.: CA/$(WWC)_2$, CA/$(YYC)_2$ CA/6F. As a reference material, the pure calcium alginate fibrous substrate was used.

The results of modification with short peptides were evaluated via scanning electron microscopy (SEM): small aggregates were observed (40-100 nm) on the surface of fibers, and the fibers size remained the same after modification (11-12 μm). The size of aggregates depended on the kind of short peptide; the smaller (40 nm) aggregates were observed when the peptide had only aromatic chain (6F), the bigger (<100 nm) ones were observed when the peptide had heterocyclic rings in the chain (WWC and YYC). All materials were contacted with osteoblast-like cells (MG-63) to test biocompatibility (cells viability after 3 and 7 days) and the results proved showed higher viability in the polysaccharide-peptide system which increased with the time of observation. The durability of polysaccharide-peptide systems was tested using the enzymatic assay: collagenase confirmed the stability of materials. The progress of degradation rate was observed using infrared spectroscopy (FTIR-ATR) - the ratio on bands with C-O and C-OH increased after degradation under in vitro conditions.

Results of the investigations on the fibrous substrates have confirmed that the system is a good model of an extracellular matrix (ECM) due to its chemical composition and microstructure which both have biomimetic characteristics. Thus, it may be used as a filling of bone defects supporting the regeneration of the damaged tissue. Additionally, it may also serve as the model research system of ECM.

Keywords: *synthetic extracellular matrix, biomimetic, peptide, polysaccharides, laboratory model*

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Introduction

The extracellular matrix is a natural microenvironment for cells of all tissues constituting a living organism [1]. The main task of the matrix is not only to provide the cells with scaffolds but also to ensure such a location that enables the cells to pick transmitted signals. The proper communication via biochemical or biomechanical signals guarantees the maintenance of homeostasis as well as the correct course of morphogenesis or cell differentiation [2-3]. From the structural point of view, the fibrous matrix is composed of proteins and polysaccharides. Protein fibres, i.e. collagen and elastin, are enriched with non-fibrous forms, such as glycosaminoglycans (GAG), proteoglycans (PG) or free matrix proteins [4]. This specific fibrous composite consisting of biopolymers (proteins and polysaccharides) is a model that scientists attempt to reproduce in the laboratory conditions. Yet, the concept of using the natural ECM framework obtained via tissue decellularization poses many problems. The difficulties include the antigens removal, the risk of rejecting the scaffold after implantation and the destruction of fibers during the chemical treatment of ECM. Another issue is the lack of process repeatability. All the mentioned problems force scientists to research alternative solutions [1]. A new strategy is designing of a scaffold that will act as an extracellular framework and imitate the scaffolding structure and microstructure (biomimetic approach) [5-6]. A good example of biomimetic ECM scaffolds are polysaccharide fibers the microstructure modified with short peptides.

Fibrous scaffolds are attractive substrates stimulating cells to faster adhesion and proliferation. The interaction between cells and materials will be even more effective when the cells encounter peptides suitable for creating a chemical complex between the cell's plasma and the biomaterial's surface (FIG. 1).

The purpose of the work was to develop and characterize the fibrous substrate (matrix) produced from calcium alginate modified with short peptides. The deposition of peptides on the fibers surface was conducted by the electrospraying technique. The peptides used in the experiment were hexapeptides basing on tyrosine and cysteine $(WWC)_2$, tryptophan and cysteine $(YYC)_2$ or phenyloalanine (6F). The polysaccharide-peptides systems were subjected to both microstructural (via optical microscope, scanning electron microscope) and structural examinations (FTIR-ATR). Then the durability of the systems was assessed in the external environment of water and enzymes (collagenase). The tests revealed that all CA/peptide systems are characterized by microstructure stability (SEM), the high degree of soaking and durability during degradation processes (FTIR-ATR). Thus, they are a good research model to conduct experiments involving cells from continuous lines (MG-63).

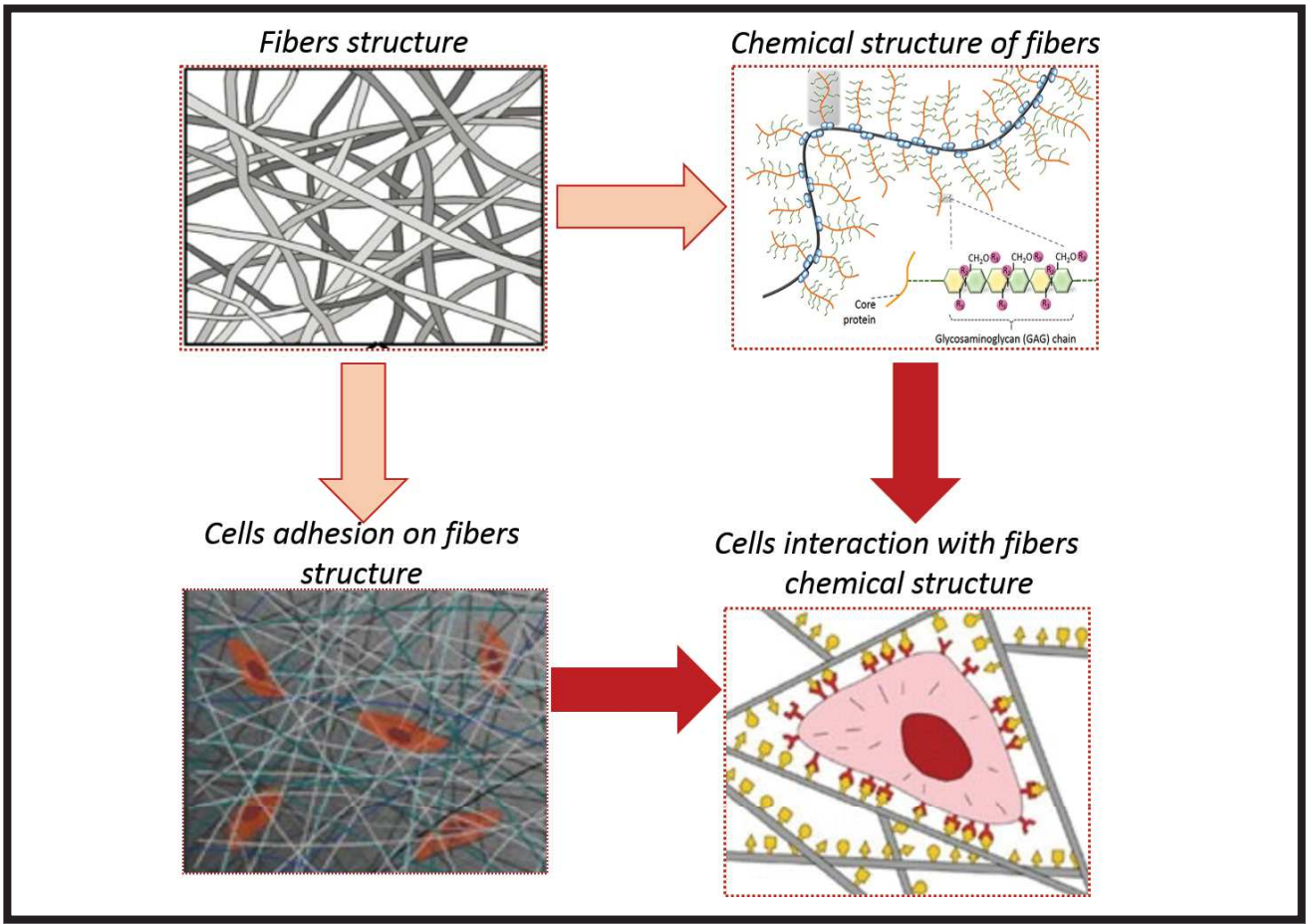

FIG. 1. The strategy of biomimetic, fibrous scaffolds with protein stimulated cells-materials interaction.

Materials and Methods

The polysaccharide fibrous substrate made of calcium alginate was obtained by the method of extraction solution at the Faculty of Technology and Textile of the Lodz University of Technology. According to the methodology of this process, sodium alginate (Protanal 80/20, BioPolymers) was dissolved and homogenized to obtain a spinning solution. Next, the solution was passed through the spinneret directly into the precipitation bath (3% solutions of $CaCl_2$/NaCl). Further, the calcium alginate (CA) fibers were washed with ethyl alcohol solutions of various concentrations (50, 70 and 90%) in order to remove residues of the precipitation bath. The short peptides were synthesized at the Institute of Organic Chemistry of the Lodz University of Technology. The first hexapeptide consisted of tryptophan (W) and cysteine (C), the second series of peptides included tyrosine (Y) and cysteine (C) and the third peptides contained phenylalanine (F). Some important data on the short peptides were presented in TABLE 1.

Due to solubility of all the peptides in the 70% solution of ethyl alcohol, the 0.5% w/wt. peptides solutions were prepared. In such conditions, the peptides size distribution was measured using the DLS method (Litesize 500 DLS, Anton-Paar). The wettability of all the tested materials was characterized (DSA 25, Kruss). Ethyl alcohol (EtOH, Avantor SA) was used in this method as a liquid. The polysaccharide fibrous substrate was characterized by a swelling test, during which the alginate fibers were immersed in water at 37°C for 24 h. The mass changes and fibers size (SEM, Nova NanoSEM) were observed after the swelling. Finally, using the electrospinning method (the apparatus settings: voltage 15 kV, application time 10 min, chamber temperature 37°C) – the peptides solutions with alcohol (1% w/wt.) were applied. In this manner, a series of the substrates based on calcium alginate fibers modified with three different hexapeptides was developed for the tests. The calcium alginate fibers were selected as the polysaccharide reference material. The microstructural characteristics of the initial and the modified fibers was performed using SEM microscopy (Nova NanoSEM, FEI). The polysaccharide-peptides systems were also tested for enzymatic degradation process in order to check their *in vitro* stability. The concentration of ions in the immersion medium (Ringer solution /37°C/3 months) and the concentration of collagenase (in the Tracine buffer) were monitored during the incubation [7]. The progress of the enzymatic degradation process was assessed by the infrared spectroscopy (FTIR-ATR) after the degradation process.

TABLE 1. Characteristics of short peptides: compositions, size, and wettability.

peptide	name	size, DLS [nm]	wettability, theta [°]
WWCWWC	$(WWC)_2$	112.8	39.4 ± 5.6
YYCYYC	$(YYC)_2$	391.3	38.7 ± 3.9
FFFFFF	6F	40.5	69.4 ± 5.4

Results and Discussion

The short peptides were well pre-wetted by ethyl alcohol (wetting angle about 40° for mixed hexapeptides, for a peptide composed of the same amino acids the wetting angle was about 70°). The peptides slowly and partially dissolve in an alcoholic medium and the higher solubility was obtained when they were heated to about 30°C. This was a short-term effect because the decrease in temperature below 30°C again caused the strong peptides agglomeration (as seen in the results of the particle size distribution, TABLE 1). The short peptides used in the experiment have aromatic rings in their structure (typophan - an indole ring, tyrosine - a phenolic ring) which facilitate the process of rolling back. It is known that peptide chains enriched with tyrosine or tryptophan are twisted to β-harmonic [8]. Such a phenomenon is not observed for peptides rich in phenylalanine (the smallest agglomerates in hexapeptide 6F are about 40 nm). The tested fibrous materials are supposed to serve as scaffolds implanted into the human body. Therefore, they should be characterized by a high level of water absorption. The obtained results indicated that, regardless of the kind of the applied peptide, all substrates displayed the water absorption higher than 300% (TABLE 2). This was confirmed by the hydrogel character of calcium alginate [9].

The neat CA fibers had a diameter of 11-12 μm and their surface was quite regular and smooth. The selected method of the substrates fabrication resulted in the multi-directional fiber-distribution with pores located among them. The application of the peptides did not cause significant alterations to the fiber morphology and diameter (FIG. 2). In the case of the fibers modified with hexapeptide (6F) it was very difficult to find aggregates (particles size of the peptide was about 40 nm). However, new forms appeared on the surface - the aggregates of the respective peptides (FIG. 2: CA/$(WWC)_2$, CA/$(YYC)_2$). The peptides with an indole ring (e.g. tryptophan) were more prone to form irregular stack forms or β-sheet [10-11]. On the other hand, the tyrosine-based peptides (a phenolic ring) did not show such tendencies [12], so they formed more irregular shapes.

TABLE 2. Composition and physical properties of compositions based on nonwoven fibers (calcium alginate, CA) modified with the short peptides.

Samples name	Swelling [%]	Fiber diameter, before degradation [μm]	Fiber diameter after degradation [μm]
CA	320	10.92 ± 1.28	10.54 ± 1.61
CA/(WWC)2	210	11.99 ± 1.25	11.47 ± 1.76
CA/(YYC)2	280	11.59 ± 1.39	11.28 ± 2.04
CA/6F	300	11.97 ± 2.37	11.56 ± 2.84

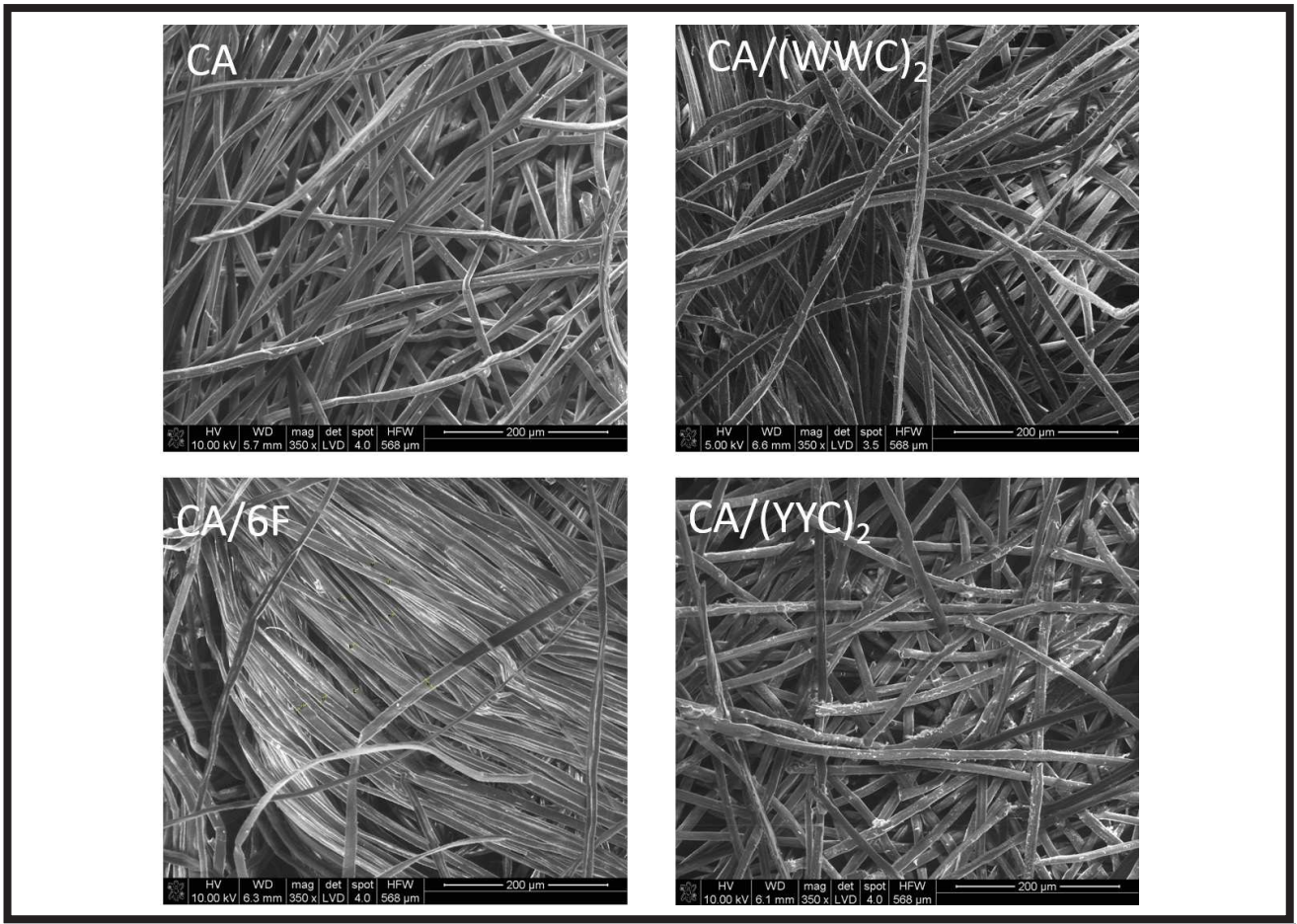

FIG. 2. The microstructure of the fibrous scaffold modified with the short peptides.

The peptides presence on the fibers was confirmed not only by the change in morphology but also by the subtle changes in the CA spectrum (FIG. 3a). The bands in the range 1100-1350 cm^{-1} proved the presence of the ring in the peptide (more visible in the first derivative). The bands ranging 3400-3600 cm^{-1} were evidence of the peptide bonds (amide 1^{o} and 2^{o}). Their intensity was relatively smaller than the intensity of the bands originating from the substrate, which resulted from a small amount of the applied peptide. A change in the matrix morphology resulted from the hydrolytic degradation conducted for 4 months in enzyms (collagenase in Tracine buffer). Namely, the fibers became rough and the aggregates of proteins were more difficult to recognize. The fibers diameters did not change (TABLE 2) but their surface was less homogeneous. Probably the polysaccharides-peptide interaction had only the electrostatic or hydrogen bond character, hence the change in the external environment caused the relaxation of these interactions. The peptides $(WWC)_2$ were able to penetrate inside the fiber, as it was evidenced by the changes in the FTIR-ATR spectra taken for the degraded materials (FIG. 3b). In the fiber itself, the bands at 1200 cm^{-1} were observed for the tyrosine systems, whereas sulphur ions (derived from cysteine) were found in the incubation solution.

The ion concentration was higher if the tryptophan-based systems (YYCYYC) or the tyrosine base system (WWCWWC) were used for the modification. The previous study showed that the hydrolytic degradation conducted in the Ringer solution (RS) resulted in dissolving the polysaccharide substrate. This phenomenon confirmed the assumption that calcium alginate fibers in the presence of cations (derived from PBS) got substituted, forming the systems of sodium alginate with salt soluble in water [9]. The examined peptides did not dissolve in the Ringer solution; therefore, they did not undergo the enzymatic degradation [13].

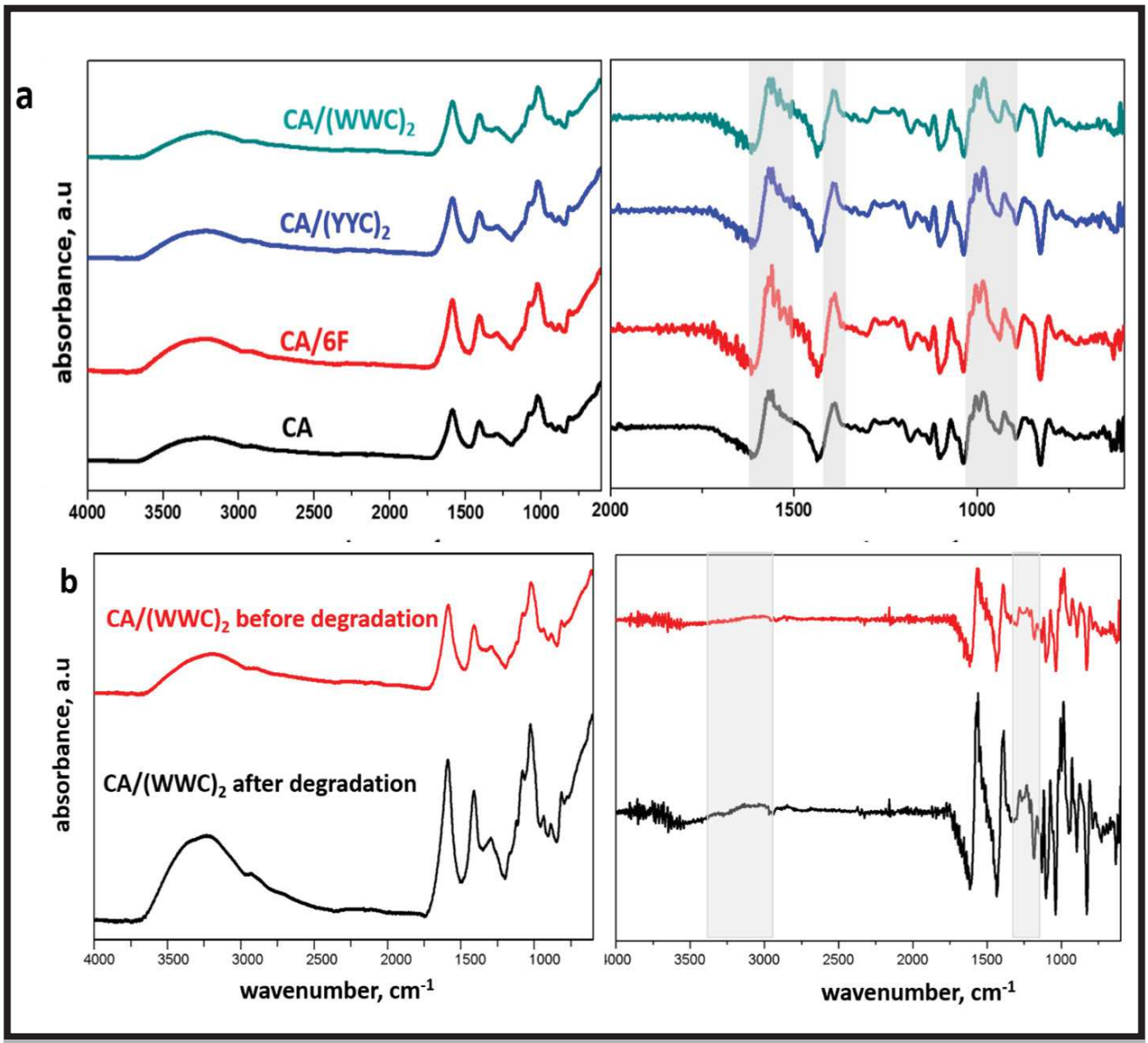

FIG. 3. FTIR-ATR spectra of polysaccharides-peptides system: (a) after modification with peptides WWC, YYC 6F (conventional spectrum, the first derivatives of spectrums), (b) after degradation $CA/(WWC)_2$ (conventional spectrum, the first derivative of the spectrum).

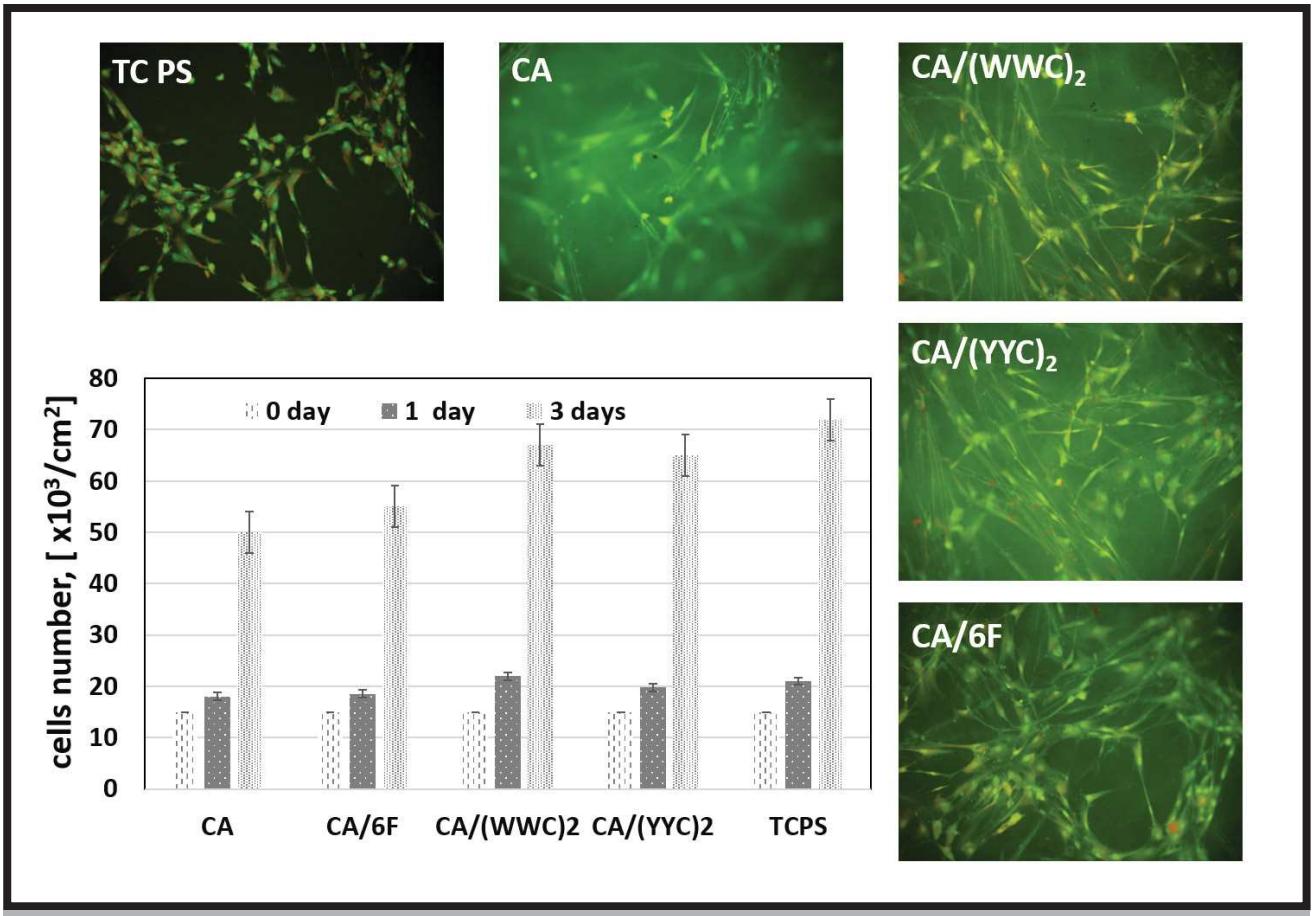

FIG. 4. Viability of MG-63 cells after 1-day and 3-days incubation and their morphology on the fibrous scaffolds observed in 3 days.

Due to their high wettability, the peptide-polysaccharides systems were not easy to conduct experiments on. The gel base made it difficult to observe the morphology of MG-63 cells. However, the quantitative determination of the cells confirmed the good biocompatibility of the tested materials after the 1- and 3-day incubation periods (FIG. 4). The most advantageous results were obtained for the fibers modified with tyrosine-based peptides. Tyrosine is an amino acid that is sparingly soluble in water, yet it is necessary for proper metabolism [14]. Introducing the additional amount of tyrosine into the system did not affect its environmental stability or cell functions (admissible amount of tyrosine in CCM is 0.029-0.197 g/L) [15]. In turn, the presence of tryptophan may influence the physicochemical properties of the substrate (decrease its wettability), supporting the metabolic processes associated with the cell spreading [16].

Conclusions

The tested protein-sugar systems derived from calcium alginate and synthetic short peptides constitute a promising group of *in vitro* stable substrates.

Taking into account the morphology and structure, the calcium alginate modified with the peptides $(WWC)_2$ and $(YYC)_2$ mimics the polysaccharide-peptide systems that build the extracellular matrix.

Due to their durability, the examined materials can be used as models for biological tests aimed at the microstructure improvement (making it more biomimetic).

They may also be used in further research to assess the possibilities of supporting regenerative processes.

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