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Research Article

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Bioinspired composite materials: sorbents for chromatography, bone-like structures and substrates for cell cultures

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ABSTRACT. Simulation of calcium and silicon biomineralization made it possible to obtain various composite particles containing an inorganic component and an organic polymer. These particles exist in a variety of forms, from stable aqueous dispersions to solid materials. New composite materials are promising for creating functionalized coatings and three-dimensional structures of different morphology. We prepared composites of calcium carbonate and phosphate by forming an inorganic phase in the presence of water-soluble polymers. The potential of new materials as sorbents for chromatography, 3D bone-like structures, and coatings for cell growth was demonstrated.

Keywords: calcium carbonate and phosphate mineralization, nanoparticle, polyelectrolyte, carboxylic polymer, cell culturing, thin-layer chromatography

1. Introduction

The formation of inorganic solid materials (silica, calcium carbonates and phosphates) in the presence of water-soluble polymers is a good model for the biosynthesis the skeleton elements of living organisms (Gower, 2008; Cai and Tang, 2008). Bones of mammals, fishes and birds, shells of mollusks, frustules of diatomic algae and sponge spicules are examples of highly ordered composite materials consisting of inorganic phase and biopolymers (Fig. 1). These materials are created from sub-micro- and nanometer-sized inorganic particles whose growth is controlled by organic polymers, such as highly modified proteins (Marsh, 1996; Poulsen et al., 2003). Biopolymers involved in biomineralization are responsible for the self-organization of primary particles at the micrometer level, as well as for interaction with cellular and supracellular structures in the formation of macro-sized skeletal elements (Marsh, 1996; Schulz et al., 2011; Wang et al., 2012; Gal et al., 2016). Extraction of biopolymers associated with inorganic particles is a very sophisticated process that often results in micrograms of partially destroyed material. Thus, synthetic polymers carrying functional groups similar to natural substances are a promising tool for studying biomineralization from the molecular level (Song et al., 2005).

Model polymers should contain groups capable of interacting with the corresponding inorganic phase: basic (amine, imidazole and sometimes hydroxyl or ester oxygen) in the case of silica and acid groups (carboxyl or phosphate) for calcium carbonates and phosphates (Nudelman et al., 2010; Annenkov et al., 2011; Krogstad et al., 2015; Xu et al., 2021). Inorganic particles are formed from low molecular weight

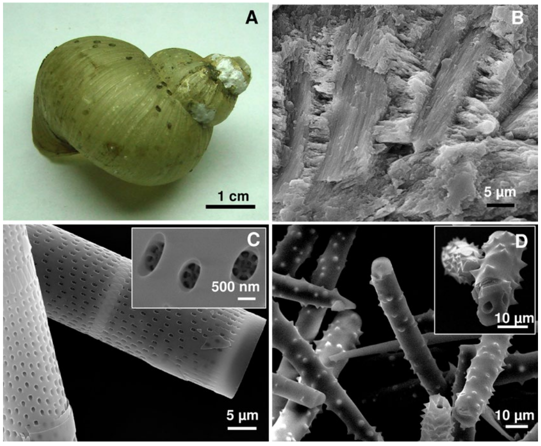

Fig.1. Photographs of mollusk *Benedictia baicalensis* (Gerstfeldt, 1859) shell and microphotographs of the shell fracture surfaces (B), diatom *Aulacoseira Sp.* (C) and spicules of sponge *Lubomirskia baicalensis* (Pallas, 1773) (D).

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precursors such as silicic acid, calcium ions, carbonate or phosphate. Depending on the polymer structure, pH, concentration and ratio of the components, etc., two main products can be obtained: composite precipitates of different morphology or aqueous dispersions, stable from a few hours to several weeks. The dispersions contain composite nanoparticles, which are considered as model primary particles in biomineralization (Sumper, 2004; Weiner et al., 2005; Nudelman et al., 2010; Annenkov et al., 2011). The next step on the way from low molecular weight compounds to skeleton elements is the transformation of composite nanoparticles into some solid material. Based on the study of living organisms and model experiments, several mechanisms of this step are discussed: an increase in the dispersion concentration (dehydration) (Grachev et al., 2008), the catalytic action of some substances (Robinson and Sullivan, 1987) or the interaction of composite nanoparticles with a polymer or nanoparticles of opposite charge (Sumper, 2004). All these mechanisms allow us to imagine the involvement of cellular and supracellular structures. On the other hand, the ordered composites obtained in the model experiments are interesting materials for various applications. Some of these applications are discussed in this work.

2. Materials and methods

2.1. Chemical reagents

1-Vinylimidazole (VI), acrylic acid (AA), N-vinylformamide (VFA), poly(vinyl alcohol) 72 kDa, azobisisobutyronitrile (AIBN), allyl glycidyl ether (AGE), rhodamine B, rhodamine 101, tetrachloroethylene, methanol, dioxane, $CaCl_2 \cdot 6H_2O$, Na_2CO_3, $(NH_4)_2HPO_4$, NaCl, 0.1 M HCl and NaOH, were of reagent grade (Merck, Fisher, or Acros Chemicals). Deuterium oxide (D_2O, 99.8 atom D%) was purchased from Sigma-Aldrich. VI, AA, and VFA were purified by distillation at reduced pressure. Sodium acrylate (AA-Na) was obtained by mixing ethanol solutions of AA and NaOH and the resulting precipitate was separated by filtration, washed with ethanol, and dried in vacuo. Dioxane and ethanol were purified according to (Keil et al., 1966). AIBN was recrystallized from ethanol. Thermo Scientific SnakeSkin Dialysis Tubing (3.5 K MWCO, 22 mm) was applied for the dialysis. Sartorius syringe membrane filters 0.45 μm (cellulose nitrate) were used for filtration. Deionized water (resistivity 18.2 MΩ·cm) was used from a purification system of Millipore Simplicity UV (USA).

2.2. Synthesis of polymers

Copolymers 1-vinylimidazole-co-acrylic acid (VI-AA) were synthesized by radical polymerization according to (Danilovtseva et al., 2004). The content of the VI units in the copolymers was determined by UV spectroscopy similarly to (Kuznetsov et al., 2017). Poly(vinyl amine) (PVAm) was obtained by alkaline hydrolysis of poly(vinyl formamide) (Pavlov et al., 2004). Vinylamine-co-acrylic acid (VAm-AA), 1-vinylimidazole-co-vinylamine (VI-VAm) and 1-vinylimidazole-co-vinylamine-co-sodium acrylate copolymers (VI-VAm-AA-Na) were prepared by radical copolymerization of vinyl formamide with further hydrolysis according to (Danilovtseva et al., 2022). Poly[N- (3-((3-[dimethylamino]propyl)(methyl)amino) propyl)-N-methylacrylamide] (PAN3) was synthesized according to (Danilovtseva et al., 2017). Poly (vinylphosphonic acid) (PVPA) was prepared according to (Millaruelo et al., 2008).

2.3. Synthesis of composite materials

Sorbent (MC25-21) for thin-layer chromatography (TLC) was prepared by coprecipitation using stock solutions of Na_2CO_3 (24 mM, pH = 9) and $CaCl_2$ (24 mM), in the presence of VI-AA copolymer (56.4% acrylic acid, 24 mM, pH = 9). Concentrations of copolymers and $CaCO_3$ in final dispersion were 6 mM. The precipitate that formed after 24 hours was separated by centrifugation (3000 g, 5 min), washed with water (4°C, pH 9), and freeze-dried.

Stable dispersion of composite calcium phosphate nanoparticles (MC20-21) for sorbent modification was prepared by mixing stock solutions of $(NH_4)_2HPO_4$ (24 mM, pH = 10), $CaCl_2$ (24 mM) and VI-AA copolymer (56.4% acrylic acid, 24 mM, pH = 10) in ratios 1.67: 1: 1 respectively.

Macroporous composite calcium phosphate materials were prepared by coprecipitation using stock solutions of $(NH_4)_2HPO_4$ (24 mM, pH = 10), $CaCl_2$ (24 mM), in the presence of VAm-AA copolymer (24 mM, pH = 10). The ratios of VAm-AA copolymers: Ca: PO_4 were 6: 6: 3.6 mM for VAm-AA with 48% AA (MC-C5) and 12: 6: 3.6 mM for VAm-AA with 40% AA (MC-D4) and 62% AA (MC-D5). The precipitate that formed after 24 hours was separated by centrifugation (3000 g, 5 min), washed with water (4°C, pH 10), and freeze-dried.

Functionalized composite coatings were prepared by mixing stable dispersions of calcium carbonate and copolymers with PAN3, PVAm (polymerization degree 163) or stable dispersion of organo-silica nanoparticles (Table). Stable dispersions of calcium carbonate with polymers were prepared by mixing stock solutions of Na_2CO_3 (24 mM, pH = 9) and $CaCl_2$ (24 mM), in the presence of copolymers (24 mmol, pH = 9). Concentrations of copolymers and $CaCO_3$ in final dispersion – 6 mM. Stable dispersion of organo-silica nanoparticles was prepared according (Annenkov et al., 2011) at pH 7 with VI-VAm copolymer, concentrations of silica and copolymer in final dispersion - 10 mM. Resulting suspensions for coating were diluted 4 times 2 minutes after mixing and applied to coverslips.

PV21-R52 coating was prepared by applying the composition PVAm-AGE (10% AGE from PVAm, concentration 0.33%) on glass with further drying and heating at 90-100 °C during 45 min.

PV21-R51 coating was prepared by applying an excess of 0.1 M polyvinyl phosphonic acid solution to PV21-R52 for 2 minutes, after which the surface was washed with water, carefully blotted with filter paper, and dispersion of organo-silica particles was coated.

Table. Preparation of composite coatings by mixing negatively and positively charged nanoparticles.

Samples	"Negative"	"Positive"	Relationships of Neg : Pos polymers, mM	Dispersion loading, µL/cm^2
CaP-91-21-p	VI-AA(73.2)-Ca	PAN3	4 : 8	20
CaP-93-21-p	VI-AA (84.5)-Ca	PAN3	4.8 : 4.8	20
CaP-96-21-p	VI-VAm-AA(75)-Ca	PVAm	3 : 12	20
PV21-R50	VAm-AA(48.4)-Ca	VI-VA-Si pH 7	3 : 5	40
PV21-R51	Sandwich PVAm-PVPA	VI-VA-Si pH 7	-	50
PV21-R52	Glass slide coated with PVAm		-	7.5

2.4. Cell growth on the composite coatings

Nonmalignant human postnatal fibroblasts (HPF) obtained *via* lentiviral transduction of the full-length TERT gene (donated by Dr. E. Dashinimaev, Engelhardt Institute of Molecular Biology, Moscow) were used to observe cell adherence and proliferation.

The modified coverslips were treated with 96% ethyl alcohol and placed in 6-well plates and the plates were sterilized under UV light for 30-40 minutes. Remaining ethanol was removed by washing with phosphate buffer solution.

Fibroblasts in the amount of 100,000 per well were seeded in wells with modified coverslips, and incubated in DMEM medium supplemented with 10% fetal bovine serum, gentamicin (50 µg/ml) at 37°C, in a humid atmosphere containing 5% CO_2. After 4 days, to evaluate the proliferation and cytotoxicity of the coatings, the cells were stained with Hoechst-33342 cell nucleus dye and propidium iodide, which stains only dead cells.

2.5. Instrumentation

SEM was performed using FEI Quanta 200 instrument. The dried composite precipitates and coatings were placed on aluminum sample holders (either on double-sided adhesive tape or dispersed in dioxane and dripped onto the plate), then coated with gold using the SDC 004 coating machine (BALZERS).

The absorbance spectra were measured on an SM-2203 spectrofluorometer (CJSC Spectroscopy, Optics and Lasers – Modern Developments (SOLAR), Minsk, Republic of Belarus). IR spectra were recorded on an Infralum FT-801 instrument (SIMEX company, Novosibirsk) using KBr pellets. Fluorescence microscopy was carried out using a MOTIC AE-31T MOTIC AE-31T and Leica DMi8 inverted microscopes. Ultraviolet analytical cabinet "UFK-HD" (LLC "PETROLASER", St. Petersburg) was applied to detect chromatographic plates.

3. Results and discussion

3.1. Sorbents for chromatography

Precipitation of calcium carbonate in the presence of 1-vinylimidazole-acrylic acid (VI-AA) copolymer yields spherical particles with a diameter of several microns (Fig. 2). These particles consist of small crystals of calcite and contain 3% organic polymer (Danilovtseva et al., 2022). Using 0.45% poly(vinyl alcohol) as a binding agent, we prepared glass plates for thin-layer chromatography (TLC). Chromatography of a mixture of rhodamine B (Rhod-B) and rhodamine 101 (Rhod-101) (Fig. 2) showed the ability of the composite sorbent to separate such similar compounds as these rhodamines.

This rhodamine model mixture separates poorly with 10-60 µm silica gel for flash chromatography (Fig. 3). Modification of the silica gel with poly(vinyl amine) and dispersion of composite calcium phosphate nanoparticles resulted in a sorbent effective for the rhodamine separation.

3.2. Macroporous material for regenerative medicine

Human bones consist of macroporous composite material mainly from calcium phosphate (hydroxyapatite), collagen type I and noncollagenous proteins (Boskey, 2013). There are various approaches to

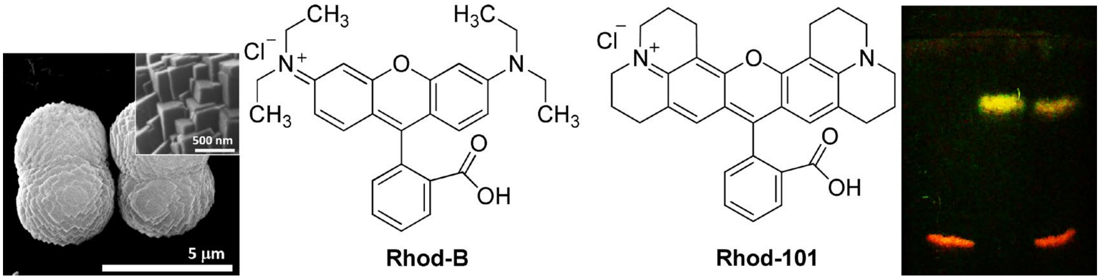

Fig.2. SEM images of calcium carbonate obtained in the presence of VI-AA copolymer (MC25-21), rhodamine formulas, and a chromatogram of a 1:1 mixture of Rhod-B and Rhod-101. The left line on the chromatogram is Rhod-101, the center line is Rhod-B, and the right line is the mixture of rhodamines. Eluent – tetrachloroethylene/methanol 7.33:1 mixture. Excitation with 365 nm light.

bone tissue regeneration, from therapeutic activation of tissue growth to creation of three-dimensional bone-like artificial structures (Dimitriou et al., 2011; Donnaloja et al., 2020). Structure of composite materials based on calcium phosphate (Fig. 4) is similar to morphology of human bone tissue (Midura et al., 2007; Chappard et al., 2011; Kytyr et al., 2012).

3.3. Functionalized composite coatings

Various high-tech applications require coatings containing active functional groups in high concentrations. Chemical and biological sensors, including DNA/RNA biochips, are created by grafting sensor molecules onto surfaces (Fixe et al., 2004). The high surface concentration of active groups can be achieved by three-dimensional placement, in a hydrogel (Timofeev et al., 1996) or on polymer chains attached to a “hilly” nano-structured surface (Annenkov et al., 2005). Many cellular technologies, including regenerative medicine, cell culture production, and the study of cell structure and function, require immobilization of cells on surfaces of various configurations.

Stable dispersions of calcium-containing composites contain nanoparticles of negative charge (Danilovtseva et al., 2022). On the other hand, polymeric amines and composite nanoparticles from silica and polymeric amines are positively charged (Annenkov et al., 2011). The interaction of these dispersions with each other can yield modified glass slides with a “hilly” surface and a positive surface charge (Fig. 5). The surface density of the amine groups on these surfaces was measured by staining with picric acid and is 500 nm^{-1}. This high content of amine groups is due to the heterogeneity of the surface and the presence of functional groups in each unit of the organic polymer. Experiments with fibroblasts showed the ability of cells to attach to new coatings and high cell viability (Fig. 6).

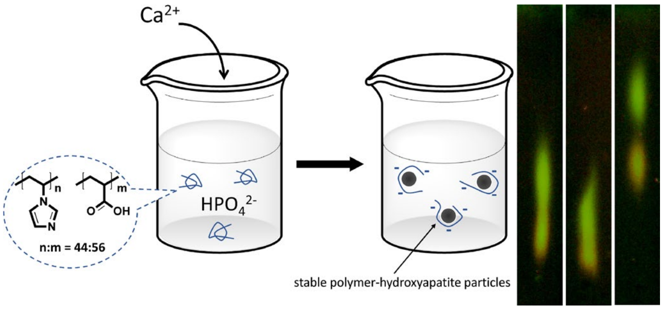

Fig.3. Scheme of synthesis of the dispersion of composite calcium phosphate nanoparticles (MC20-21) and a chromatogram of a 1:1 mixture of Rhod-B and Rhod-101. The left plate is 10-60 μm silica gel for flash chromatography, the center plate is the silica gel modified with poly(vinyl amine), and the right plate is the silica gel modified with poly(vinyl amine) and dispersion of composite nanoparticles. Eluent – methanol. Excitation with 365 nm light.

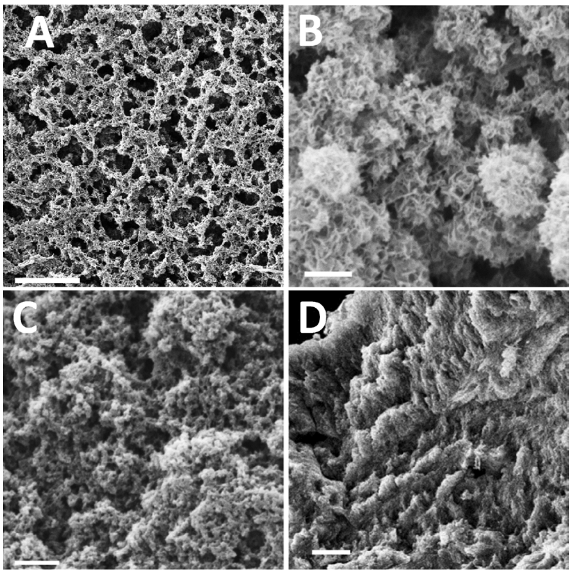

Fig.4. SEM images of calcium phosphate obtained in the presence of VAm-AA copolymers: A - MC-C5; B - MC-D4; C – MC-D5; and D - a human lower jaw bone sample (Kharitonov et al., 2014). Scale: A and D – 10 μm, B and C - 1 μm.

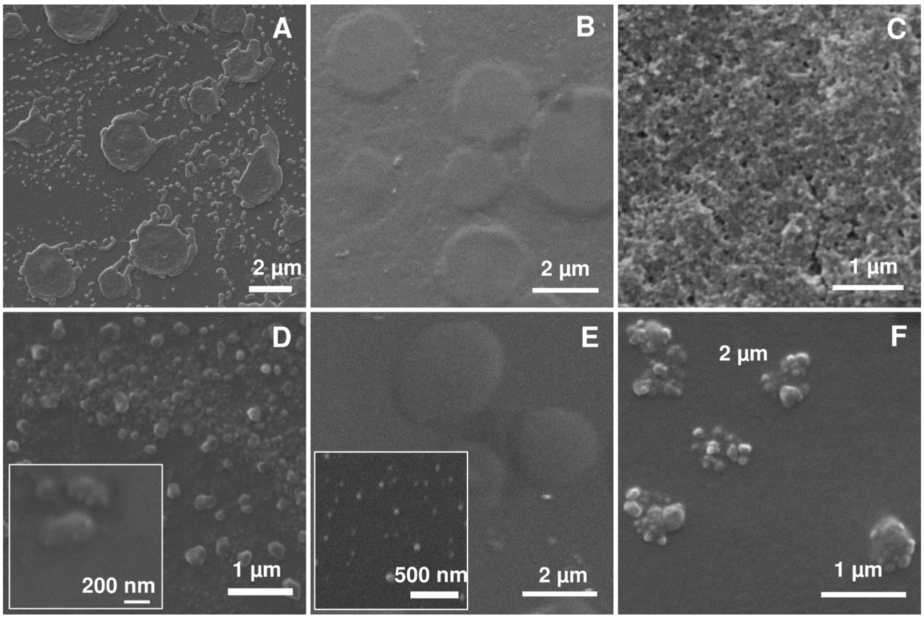

Fig.5. Scanning electron microscopy images of the coatings obtained from aqueous suspension of composite precipitates. A - CaP93-21-p, B - CaP93-21-p coating after 7 days in phosphate buffer solution pH 7.4 at 37°C, C - PV21-R50, D - PV21-R51, D insert - PV21-R52, E - CaP96-21-P, F - CaP91-21-P.

4. Conclusions

Thus, composite bioinspired particles obtained by precipitation of calcium carbonate or phosphate in the presence of organic polymer are a promising source for designing new materials. Micrometer-sized particles can be used as a sorbent for chromatography. In some cases, precipitation results in three-dimensional macroporous materials whose morphology resembles that of mammalian bones, which is of interest for regenerative medicine. Stable aqueous dispersions of composite nanoparticles are a source for creating coatings for cell culturing.

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Conflict of interest

The authors declare no conflict of interest.

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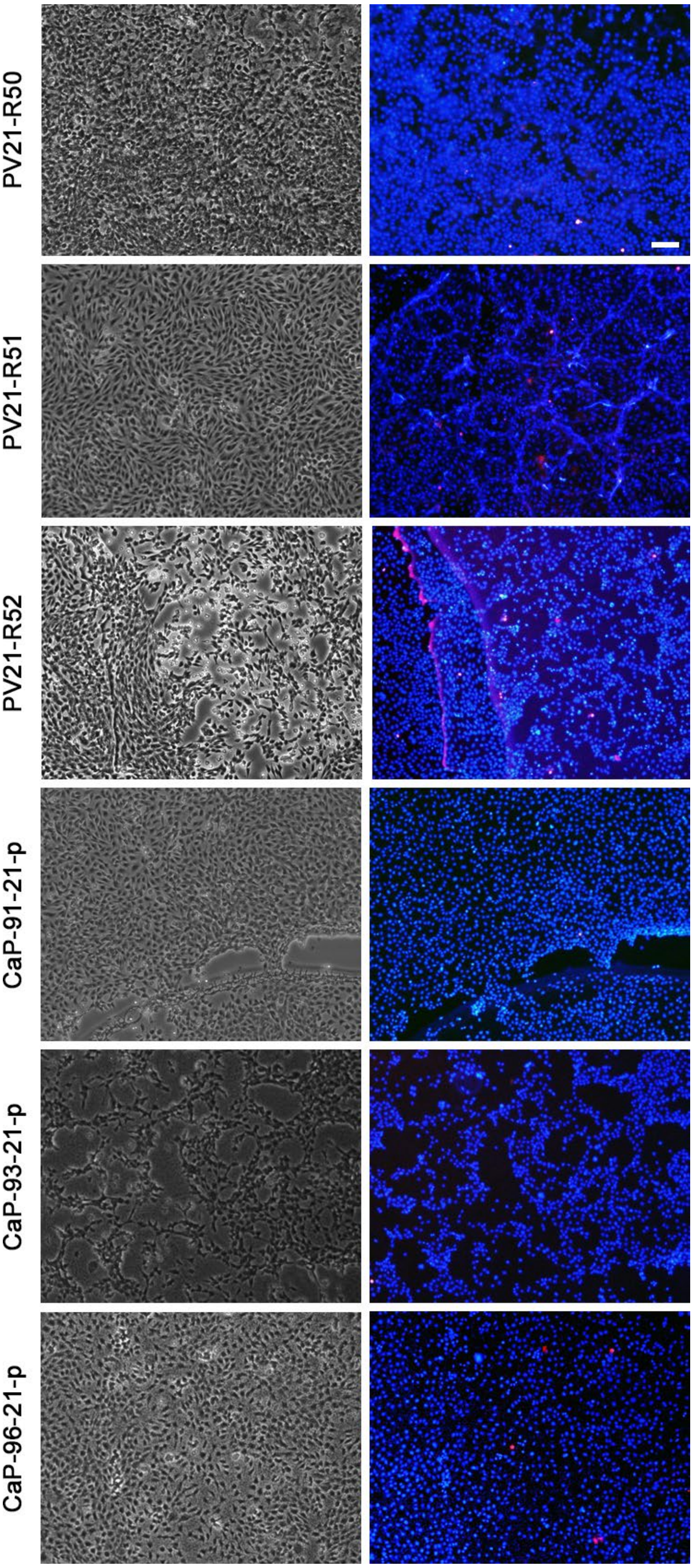

Fig.6. Phase contrast (left) and fluorescent (right) images of fibroblasts growing of modified coverslips. Blue fluorescence corresponds to live cells stained with Hoechst-33342; pink fluorescence corresponds to dead cells stained with propidium iodide. Scale bar represents 100 μm.

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