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SYNTHESIS AND INVESTIGATION OF MODIFIED SILICA COATINGS FOR BIOTECHNOLOGY

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Mesoporous organic-inorganic hybrid composites on glass substrates were prepared by the sol-gel method for testing the proteins adhesion. Different types of hydrophobic/hydrophilic silica sol-gels were prepared using tetraethoxysilane (TEOS) as starting material and modified with hexamethyldisilazane (HMDS). Sol-gel thin films were successfully prepared with the dip-coating technique on glass surfaces. The coatings surface characteristics were evaluated. The prepared sol-gel derived colloidal silica coatings and modified coatings were characterized by wettability measurements. Also, infrared spectroscopy, atomic force microscope (AFM) assay were used to characterise the surfaces. The coatings of colloidal silica (VT104, water contact angle 17°), polysiloxane sol (VT111, 64°) methyl-modified sols (VT079, 144° and VT112, 47°) with various wettability properties were tested for CaCo-2 cells proliferation. Methyl-modified coating VT112 proved to be the best substrate for cell proliferation.

INTRODUCTION

A combination of sol-gel compounds and biomaterials like bio-molecules, cell, bacteria, viruses and etc. resulting in added functionality. Bio-doped hybrid materials provide a unique opportunity for physicists, chemists, biologists and material scientists to mould the new area of nanobiotechnology [1, 2]. Organic and biological materials combination with the sol-gel matrices could be used for various applications: in biomolecular electronics, biosensors, bio-actuators and medicines, namely in photodynamic anticancer therapy, targeted delivery of radio isotopes, drug delivery, electronic DNA sequencing, nanotechnology of gene delivery system and gene therapy [1–7].

Bio-molecules are naturally occurring molecules in living organisms, e.g., amino acids and nucleotides, consisting primarily of carbon, hydrogen, oxygen, nitrogen, phosphorus, and sulfur. Various biological molecules adhesion and growth on the sol-gel matrix is influenced by bioactive surface properties as type and the density of surface charge, balance between the hydrophilicity and the hydrophobicity on surface, the chemical structure and functional groups, surface topography and roughness, the interfacial free energy, etc. [6]. The surface structure must be similar to the biological molecules properties, consequently inorganic surfaces are modifiable with substrates which enhances interaction: organic materials, proteins, antibodies, antigens, polymers, etc. [1–7]. Thus, the essential requirements for the formation of surface structures are the possibility to modify, reiteration of the results, method must be simple and low-cost. The sol-gel derived inorganic matrices (films, micro-spheres or fibers) offer several advantages compared with organic polymers such as mechanical strength, non-toxicity or chemical inertness, so they may be used in applications where biocompatibility and/or thermal stability requirements are essential. During this method is available to use organic and inorganic compounds, it is possible to synthesize a various substances: powders, thick, thin or multilayer films, ceramic structures, nanoporous organic-inorganic membrane materials, etc. [8–14]. Moreover, the sol-gel matrix with the large surface area, porosity provides the advantages of optical transparency, good compatibility and large immobilization capacity. Sol-gel entrapment method not only improves the resistance of bio-molecules to thermal and chemical denaturation but also increases the storage stability. In the past few years, numerous silica- and/or

porous siloxane based organic–inorganic materials have been employed to immobilize or capsulate proteins, enzymes, polysaccharides, nucleic acids, phospholipids, as well as the cells hybrid materials [1–6, 15]. Hybrid sol-gel films were developed for tissue-derived cell growth [7]. It was found that immobilized biological materials trapped within sol-gel glasses usually retain their catalytic activity and can even be protected against degradation [4, 5, 7, 8, 10, 11]. Bioactive coating is also applicable for detection of viruses and for the generation of anti-viral vaccines [4].

This report presents investigations of the formation, modification and characterization of different silica-based mesoporous organic–inorganic hybrid materials on glass substrates which could useful for the adhesion or growth of bio-molecules have done. Different amount of methyl groups were introduced onto the colloidal silica to get surfaces with miscellaneous properties. In further investigations, modified and protein coated sol-gel surface with different wettability properties have been tested for CaCo-2 cell proliferation.

EXPERIMENTAL

Preparation of Sols. The precursor of SiO_2 (A sol) colloidal sol was prepared by the base catalyzed hydrolysis of tetraethylorthosilicate TEOS (TEOS, $C_8H_{20}O_4Si$, ≥98%, Fluka) by the following method of preparation of Stöber silica [16]. The ammonia solution in ethanol was added to the solution of TEOS in ethanol with continuous stirring at room temperature 20±2°C (TEOS:NH_3:H_2O:EtOH molar ratio 1:0.2:2.37:38, respectively). The molar ratio of ammonium hydroxide to alkoxide was 0.2 mol, to water – 0.4 mol. The solution with final silica concentration of 3% was prepared. The obtained reaction mixture was stored for 4 days at room temperature to allow hydrolysis as much as possible. The final product consisted of colloidal suspension of SiO_2 nanoparticles in an anhydrous solvent. Polysiloxane (PS) 3% (B sol) sol was obtained using acid hydrolysis of TEOS (TEOS:HCl:H_2O:EtOH molar ratio 1:0.01:4:37.4) in ethanol.

Methyl-modified SiO_2 sols were prepared by adding different amount of hexamethyldisilazane (HMDS, $C_9H_{19}NSi_2$, 98 %, Aldrich) to the 3% colloidal silica suspension. HMDS modified sols were aged 2 days (SiO_2:HMDS:EtOH molar ratios: C1 sol 1:0.16:44.05 (0,625 % HMDS); C2 sol 1:0.32:44.59 (1.25 % HMDS); C3 sol 1:2.5:45 (10 % HMDS)) at room temperature.

Preparation of Coatings. Coatings were prepared on glass (Menzel-Glaser, 76x26 mm) substrates previously cleaned and dried. Dip-coating method on both sides of the glass was employed to produce sol-gel coatings using apparatus KSV Instruments Ltd. KSV D™. The parameters of dipping were as following: immersion rate – 40 mm/min and dipping time – 20 s.

Characterization of Sols and Coatings. IR spectra of the materials were recorded using ATR Perkin-Elmer Spectrum BX FT-IR spectrometer. The AFM images of the silica coatings on glass were performed on Multimode Scanning Probe Microscope (Digital Instruments). For the characterization of surface properties, the measurements of water, dimethylformamide (DMF) and ethylene glycol (MEG) contact angle on KVS Instrument CAM 100 were recorded.

Cell culture. CaCo-2 cells from American Type Culture Collection (ATTC) were grown in advanced RPMI 1640 culture medium (Gibco, 12633-012) supplemented with 5% fetal bovine serum (FBS) (Gibco, 10106-169) and L-glutamine (2 mmol/l) without antibiotics, at 37°C in a CO_2 incubator (5% CO_2, 95% air, 95% relative humidity). FBS was heat inactivated prior to use (at 56°C for 30 min with gentle shaking). FlexiPERM micro 12 wells (Vivascience IV-50011436, growth area 0.3 cm^2) were stacked on microscope slides and VT079, VT104, VT111 and VT112 sample slides. The seeding number of cells was $1.5x10^4$ cell/ cm^2.

Protein coatings. *Laminin-1* from basement membrane of Engelbreth-Holm-Swarm mouse sarcoma (Sigma, L2020) was used. It was slowly thawed in the refrigerator and diluted in Hank's balanced salt solution (HBSS, Gibco, 14060-040). The surface was coated with a minimal volume (18 μl) of working solution (0.1 mg/ml) and left for 45 min. Excess fluid was sucked off and the surface was left to air dry before introducing the cell suspension. *Fibronectin* (Sigma F1141) solution in PBS (0.2 mg/ml) was used for coatings. A minimal amount (11 μl) of fibronectin solution was added to each well and left to dry for 45 min. Excess fluid was sucked off. The coated surface was rinsed with culture medium before the cell suspension was added. *Collagen-1* (Sigma C7661) was dissolved in acetic acid (0.1 mg/ml). A minimal amount (18 μl) of colla-

gen solution was placed in each well and the collagen was allowed to bind for two hours at 37ºC. Excess fluid was sucked off and the surface was left to air dry. The coated surface was rinsed with HBSS before adding the cell suspension.

Assessment of cell proliferation. Proliferation of cells was measured with the colorimetric cell proliferation BrdU (5-bromo-2-deoxyuridine) test (Kit No. 1 647 229, Roche) two days post-seeding on coated and non-coated VT samples and glass. The supernatant was replaced by 100 µl of fresh growth medium and 10 µl BrdU labelling solution was added. The cells were incubated for 90 min at 37ºC. After removal of the supernatant, 150 µl of FixDent solution was added and the cells were left at room temperature for 30 min. The supernatant was sucked off and 75 µl of anti-BrdU-POD was added. The cells were then stored for 90 min at room temperature. Afterwards, the cells were washed three times with 150 µl washing buffer. 75 µl of substrate solution was added and left for some minutes protected from light for the colour to develop. 75 µl of the solution was transferred to 96-well microtiter plates and 20 µl of 1M sulphuric acid was added. The absorbance was measured at 450 nm. Data were derived from three independent experiments and presented as means with standard deviations. The differences were analyzed using Student's *t* test on two populations and One-way ANOVA; $p<0.01$ was considered significant.

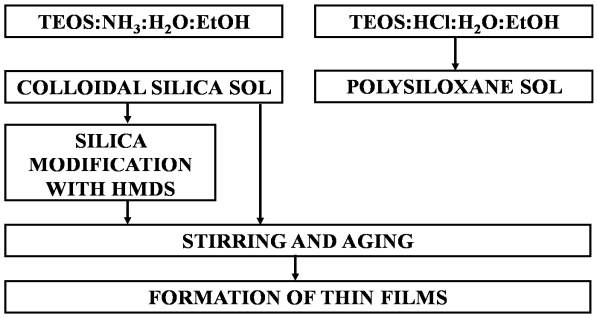

Fig. 1. Hybrid sol-gels coatings with different surface properties synthesis scheme

RESULTS AND DISCUSSION

During the immobilization of various biological molecules on the sol-gel matrixes particular attention is paid to the bioactive surface properties (density of surface charge, hydrophilicity or hydrophobicity, the chemical structure and functional groups, surface topography and roughness, the interfacial free energy, etc. [6]). Using sol-gel synthesis method it is possible to prepare materials with small pores ($d<0.4$ nm) and high specific surface area ($S_{BET}>250$ m^2/g), different chemical structure, etc. [3–6]. Total sol-gels coatings with different surface properties synthesis scheme is shown in Fig. 1.

Characterization of the Silica Hybrids. *Contact angle on the coating films.* Surface properties that influence protein adsorption and subsequent cell adhesion include surface free energy, roughness and chemistry [17]. The prepared sol-gel derived colloidal silica coatings and modified coatings were characterized by wettability measurements using the contact angle measurements because hydrophilicity/hydrophobicity and surface free energy (surface tension) is an important thermodynamic characteristic of the surface of liquids and solids. Solids with low surface free energy hydrophobic and hydrophilic with solids with high-energy [18]. One of the most frequently used methods of contact angle assessments is the sessile drop technique and the surface free energies were estimated from the contact angles. Fig. 2 shows water drop on the different coating films. Sol compositions and data of water, dimethylformamide (DMF) and ethylene glycol (MEG) contact angles of coatings are shown in Table 1.

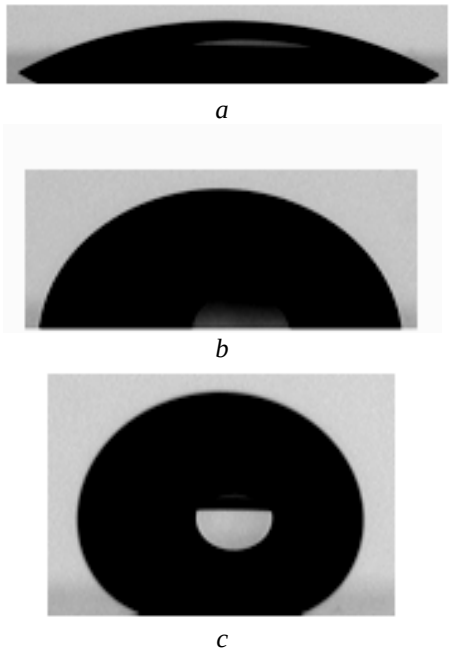

Fig. 2. Imagines of water drop on the differente coating films: *a* – SiO_2 3% (θ=17°), *b* – PS 3% (θ=64°), *c* – HMDS modified SiO_2 (θ=144°)

Table 1. Sol composition and data of H_2O, DMF and MEG contact angles, surface free energies of coatings

Sample No.	Sol composition	Contact angle (θ),°			Surface free energy γ_s, mN/m
		H_2O	DMF	MEG	
VT104	SiO_2 (3%)	17±1	11±1	30±1	71
VT111	polysiloxane (3%)	64±1	32±1	34±1	39
VT115	SiO_2 (3%)/ HMDS (0.625%)	42±1	20±1	46±1	55
VT112	SiO_2 (3%)/ HMDS (1.25%)	47±1	17±1	48±1	54
VT079	SiO_2 (3%)/ HMDS (10%)	144±1	7±1	87±1	30

The results showed that the colloidal silica surface (water contact angle 17°) is more hydrophilic and its surface free energy is lower compare to PS (water contact angle 64°). In the acid-catalyzed TEOS hydrolysis system the protonation of the alkoxide group causes electron density to be withdrawn from Si allowing the nucleophilic attack from water. In contrast, the base-catalyzed hydrolysis of silicon alkoxides proceeds through the attack of a nucleophilic deprotonated silanol on a neutral silicic acid. In general, silicon oxide networks obtained via acid-catalyzed conditions consist of linear or randomly branched polymers; by contrast, base-catalyzed systems result in highly branched clusters, so contact angle measurements and IR spectra confirms that on the SiO_2 coating film surface are more –OH functional groups than on the PS.

With the aim of finding the optimal hydrophobicity for cells adhesion and growth, the methyl-modified silica coating were prepared. The colloidal silica particles are covered by hydroxyl groups, after HMDS addition some of the hydroxyl groups are replaced by methyl groups. It can be said that the contact angle varies significantly with the mole ratio of SiO_2/HMDS by considering the accuracy. The contact angle of water and surface free energy increased with increasing amount of HMDS. In case HMDS, each monomer of HMDS consists of two trialkylsilane groups, which gets attached to the surface. The HMDS modified silica coating (VT079) show the highest contact angle (144°) and the lowest SiO_2 (VT104, 17°). The hydrophilic methyl modified silica surfaces were prepared using 0.625% and 1.25% of HMDS during modification.

FTIR Characterization. FTIR characterization of the SiO_2, polysiloxane (PS), and with HMDS modified SiO_2 hybrids is shown in Fig. 3.

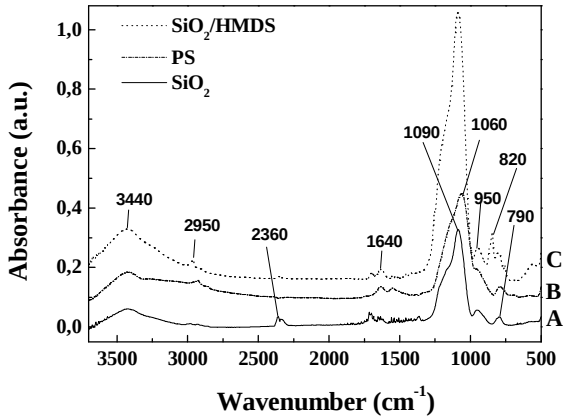

Fig. 3. FTIR spectra of the *a* – SiO_2, *b* – polysiloxane and *c* – SiO_2 modified with HMDS

The SiO_2, PS and HMDS gels exhibits hydroxyl absorption bands at 3450–3400 cm^{-1}, which arise from relatively free, non–hydrogen-bonded, and hydrogen-bonded silanols (Si–OH), respectively. The IR absorption bands at 1090 and 1060 cm^{-1} in the difference spectrums are assigned to the Si–O vibrations and 790 cm^{-1} symmetric Si–O vibrations, 950 cm^{-1} attributed to symmetric Si–OH vibrations. After the surface modification with HMDS, the intensity of absorption bands at 2950 cm^{-1} corresponding to C–H, $–CH_2–$, $–CH_3$ groups intensities are higher. The overlapped absorption bands from 800 to 1260 cm^{-1} can be attributed to SiO_2, Si–OH and organic groups (C–H, $–CH_2–$, $–CH_3$).

AFM analysis of the coating films. Coatings and the sol particles morphological studies were carried out by atomic force microscope. As can be seen from the AFM images (Fig. 4) that PS coating is rather smooth, the surface roughness is R_{MS}=0.79 nm than other coatings. The surface of SiO_2 coating roughness is R_{MS}=3.05 nm and the particles show tendency to form bigger agglomerates when SiO_2 is modified with HMDS (R_{MS} is increasing with increasing amount of HMDS). It is evident from the images that the nanosilica surface is modified by methyl groups.

Cell behaviour on biomaterial surface. The CaCo-2 cells from American Type Culture Collection (ATTC) were grown on sol-gel derived coatings with different wettability properties. The coatings obtained from colloidal silica (VT104), polysiloxane sol (VT111) methyl-modified sols (VT079 and VT112) were selected for proliferation test. The sol composition and data of water contact angles, surface free energy and surface roughness of coatings are shown in Table 2.

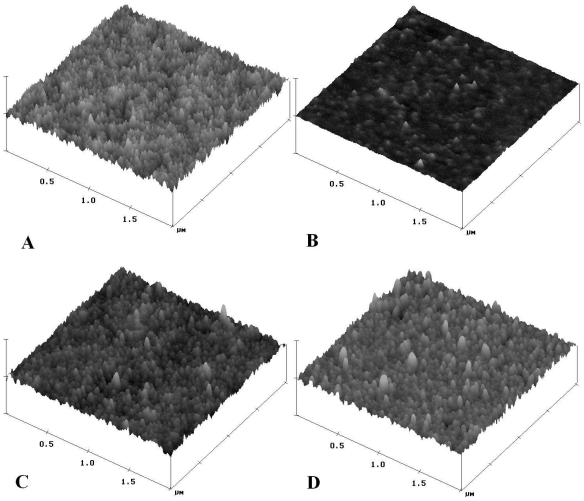

Fig. 4. AFM imagines of modified surfaces: *a* – 3% SiO2 (R_{MS}=3.05 nm, VT104); *b* – 3% polysiloxane (R_{MS}=0.79 nm, VT111); *c* – SiO2 (3%)/HMDS (1.25%) (R_{MS}=2.55 nm, VT112); *d* – SiO2 (3%)/HMDS (10%) (R_{MS}=3.27 nm, VT079)

Table 2. The sol composition and data of water contact angles, surface free energy and surface roughness of hybrid silica coatings tested for the proliferation and adhesion test of CaCo-2 cells

Sol No.	Sample No.	Functional groups on the surface	Surface roughness (R_{MS}), nm	Contact angle (θ),°	Surface free energy γ_s, mN/m
A	VT 104	–OH	3.05	17±1	71
B	VT 111	–OH	0.79	64±1	39
C2	VT 112	–OH, $–CH_3$	2.55	47±1	54
C3	VT 079	–OH, $–CH_3$	3.27	144±1	30

Cell behaviour on biomaterial surface is modulated by the concentration, composition and conformation of absorbed extracellular matrix (ECM) proteins [19]. The sol-gel coatings were coated with a minimal volume of different proteins as laminin, fibronectin or collagen-1 solutions. AFM imagines of protein coated silica surface are shown in Fig. 5 and Fig. 6.

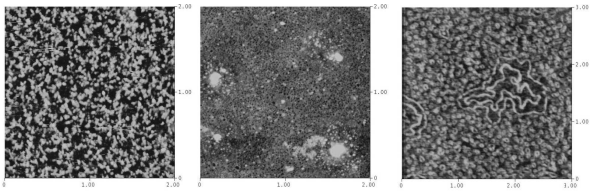

Fig. 5. AFM imagines of fibronectin coating on: *a* – glass; *b* – 3% polysiloxane (VT111); *c* – SiO2:HMDS (VT112) sol-gel modified samples

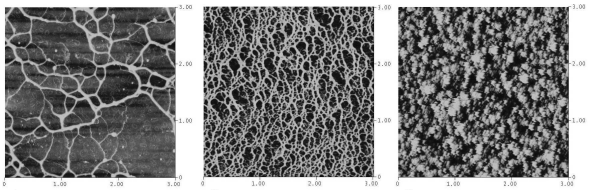

Fig. 6. AFM imagines of Collagen-1 coating on: *a* – glass; *b* – 3% polysiloxane (VT111); *c* – SiO2:HMDS (VT112) sol-gel modified samples

Results of proliferation on coatings surfaces are presented in Fig. 7 and Fig. 8.

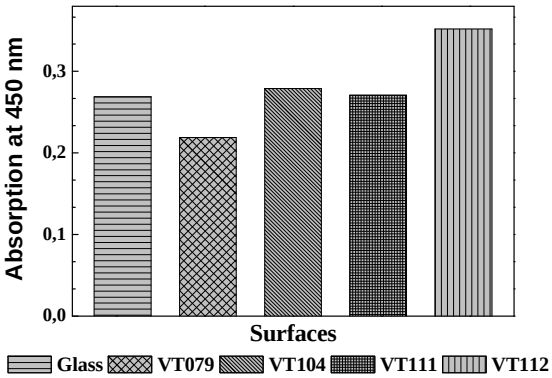

Fig. 7. Proliferation of CaCo-2 cells on VT079, VT104, VT111, VT112 samples and glass as control surface

When only simple samples (Fig. 7) are compared the means within all samples are significantly different ($p<0.01$, One-way ANOVA). Proliferation on VT079 was significantly slower and on VT112 significantly faster ($p<0.01$) then on glass control (Fig. 7). This two substrates were both methyl-modified, however contend of HMDS was 10% in VT079 and 1.25% in VT112.

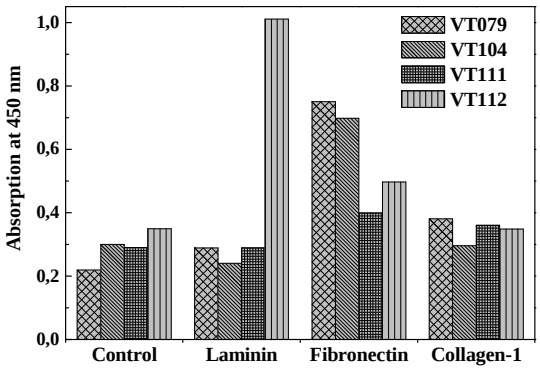

Fig. 8. Proliferation of CaCo-2 cells on laminin-1, fibronectin and collagen-1 coated VT079, VT104, VT111 and VT112 samples and corresponding controls

The means within the surfaces for all protein coated VT samples and controls are significantly different ($p<0.01$, One-way ANOVA) and almost all protein coated samples stimulated proliferation compared to the corresponding controls (Fig. 8). Laminin-1 coated VT104 sample was the only protein coated surface where proliferation was significantly slower ($p<0.01$) then on control.

CONCLUSIONS

The modified sol-gel derived silica coatings were prepared and characterized. The topography of protein coatings differs distinctly between glass and various sol-gel modified samples. The coatings of colloidal silica (VT104, water contact angle 17°), polysiloxane sol (VT111, 64°) methyl-modified sols (VT079, 144° and VT112, 47°) with various wettability properties were tested for CaCo-2 cells proliferation. Methyl-modified coating VT112 proved to be the best substrate for cell proliferation. Cell proliferation two days post seeding was significantly faster on almost all proteins coated samples compared to corresponding controls. The difference in drop contact angle between VT079 (144°) and VT104 (17°) is the biggest. From results we can assume, that surface characteristic contact angle and functional groups are significant for protein adsorption and consecutive for cell growth and proliferation. The advent of hybrid sol-gel materials opens new possibilities for tailoring efficient substrates for cell adhesion and growth. A major advantage of these materials is that the quantity of preparation methods and chemicals with which one can design almost any desired surface property is practically unlimited.

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Синтез та дослідження модифікованих кремнеземних покриттів для біотехнології

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Для тестування адгезії білків золь-гель методом приготовано мезопоруваті органо-неорганічні гібридні композити на скляних підкладинках. Різні типи гідрофобних та гідрофільних кремнеземних золів та гелів було приготовано з використанням тетраетоксисилану (ТЕОС) як вихідного матеріалу і модифіковано гексаметилдисилазаном (ГМДС). Тонкі золь-гель плівки було успішно приготовано за допомогою процедури глазурування зануренням на скляних поверхнях. Одержано характеристики поверхонь покриттів. Одержані шляхом золь-гель синтезу покриття – похідні колоїдного кремнезему та модифіковані покриття було охарактеризовано вимірюванням змочуваності. Для характеризації поверхонь було також використано аналіз за допомогою інфрачервоної спектроскопії та атомної силової мікроскопії (АСМ). Покриття з колоїдного кремнезему (VT104, кут змочування водою 17°), полісилоксановий золь (VT111, 64°), метил-модифіковані золі (VT079, 144° та VT112, 47°) з різною змочуваністю було протестовано для розмноження клітин CaCo-2. Метил-модифіковане покриття VT112 виявилось найкращим субстратом для розмноження клітин.

Синтез и исследование модифицированных кремнеземных покрытий для биотехнологии

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Для тестирования адгезии белков золь-гель методом приготовлены мезопористые органо-неорганические гибридные композиты на стеклянных подложках. Разные типы гидрофобных и гидрофильных кремнеземных золей и гелей были приготовлены с использованием тетраэтоксисилана (ТЭОС) как исходного материала и модифицированы гексаметилдисилазаном (ГМДС). Тонкие золь-гель пленки были успешно приготовлены с помощью процедуры глазурирования погружением на стеклянных поверхностях. Получены характеристики поверхностей покрытий. Полученные путем золь-гель синтеза покрытия – производные коллоидного кремнезема и модифицированные покрытия были охарактеризованы измерением смачиваемости. Для характеризации поверхностей был также использован анализ с помощью инфракрасной спектроскопии и атомной силовой микроскопии (АСМ). Покрытия из коллоидного кремнезема (VT104, угол смачивания водой 17°), полисилоксановый золь (VT111, 64°), метил-модифицированные золи (VT079, 144° и VT112, 47°) с разной смачиваемостью были протестированы для размножения клеток CaCo-2. Метил-модифицированное покрытие VT112 оказалось наилучшим субстратом для размножения клеток.