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MACROMOLECULAR COMPOUNDS AND POLYMERIC MATERIALS

Synthesis of D,L-Lactide–ε-Caprolactone Copolymers and Preparation of Films Based on Them

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Abstract—D,L-Lactide–ε-caprolactone copolymers were synthesized by polymerization in the bulk. Films of these copolymers were prepared, and their strain properties and degradation rate under physiological conditions were studied. Introduction of ε-caprolactone into the copolymer increases the film elasticity and decreases the degradation rate compared to poly(D,L-lactide) homopolymer, and also favors spreading of human keratinocytes. The films obtained can be used for treatment of wounds and burns.

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The demand of medicine for materials based on biodegradable polymers steadily increases [1]. The most demanded materials are poly(hydroxy esters). Their main representatives are poly(D,L-lactide), poly(L,L-lactide), polyglycolide, polycaprolactone, etc. [2, 3]. These polymers are nontoxic and biodegradable; in addition, by varying the synthesis conditions it is possible to obtain materials with optimum levels of mechanical characteristics and resorption rate [4, 5]. These polymers find wide use as scaffolds for cultivation and transplantation of human cells in regeneration of various organs and tissues [6–8].

Numerous studies have been performed on the development of substrates for cultivation and transplantation of human skin cells. Both natural and synthetic polymers show promise for the formation of such substrates. Among natural materials, good results were obtained with chitin and chitosan [9].

Cultivation of skin cells, keratinocytes or fibroblasts, on polymer films formed from synthetic polymers such as poly(D,L-lactide), poly(L,L-lactide), polycaprolactone, and lactide–glycolide copolymers has been reported [10, 11]. Despite successful experience in using these materials in clinical practice, biodegradable films as materials for cultivation and transplantation of skin cells have certain drawbacks [12, 13]. These drawbacks are mainly caused by specific features of regeneration of the damaged skin. In the course of skin regeneration, the film should have sufficient mechanical strength and undergo quick resorption. The resorption of poly(L,L-lactide) and poly(ε-caprolactone) films is too slow. In the case of poly(L,L-lactide), low degradation rate is caused by the crystalline structure of the polymer. In poly(ε-caprolactone), hydrophobic pentamethylene chain also prevents rapid resorption of the polymer film [14].

Films of D,L-lactide–ε-caprolactone copolymers show promise for keratinocyte cultivation. Numerous papers concerning synthesis of L,L-lactide and ε-caprolactone and properties of their copolymers have been published [15]. As already noted, the crystalline structure of L,L-lactide considerably decreases the polymer degradation rate.

Table 1. Compositions, yields, and intrinsic viscosities of the copolymers

Sample no.	Initial mixture, mol %	CL : L in monomer mixture, mol %	CL : L in copolymer, mol %	[η], dL g^{-1}	Polymer yield, wt %
1	D,L-Lactide	0 : 100	100	0.84	71
2	D,L-Lactide/ε-caprolactone 95 : 5	5 : 95	4 : 96	0.35	65
3	D,L-Lactide/ε-caprolactone 90 : 10	10 : 90	10 : 90	0.34	74
4	D,L-Lactide/ε-caprolactone 85 : 15	15 : 85	14 : 86	0.30	54
5	D,L-Lactide/ε-caprolactone 80 : 20	20 : 80	18 : 82	0.29	55

This study was aimed at synthesizing D,L-lactide–ε-caprolactone copolymers containing 5 to 20 mol % caprolactone, preparing films from them, determining their degradation rate and strain and strength characteristics, and evaluating the prospects for using these films for keratinocyte cultivation and transplantation.

EXPERIMENTAL

D,L-Lactide (Acros Organics) was preliminarily recrystallized from benzene and dried in a vacuum desiccator over paraffin turnings.

ε-Caprolactone (Aldrich) was purified by distillation from CaH_2 under reduced pressure (T_b =130–133°C, p = 30 mm Hg). Chloroform (Vekton) and methylene chloride (Komponent-Reaktiv) were distilled from P_2O_5 at atmospheric pressure. Benzene (chemically pure grade, Ekos-1) was distilled from sodium metal. Tin 2-ethylhexanoate (Sigma–Aldrich) was used as polymerization initiator without preliminary purification. Toluene (ultrapure grade, Reaktiv) and methanol (chemically pure grade, Vekton) were also used without additional purification.

The following chemicals were used for isolation and cultivation of keratinocytes: phosphate buffered saline (PBS), pH 7.2; collagenase (OAO Tekhnologiya, St. Petersburg); dispase (Roche Diagnostics GmbH, Mannheim, Germany); trypsin solution and Versene solution (Biolot, St. Petersburg); fetal bovine serum (FBS, HyClone, the United States); DMEM + $F1_2$ (3 : 1) medium for keratinocyte cultivation (Lonza, the United States); Formalin (Sigma–Aldrich, the United States); Triton X-100 (Sigma–Aldrich, the United States); Rhodamine phalloidin (Thermo Fisher Scientific, the United States); DAPI (Invitrogen, the United Kingdom); and embedding medium (Invitrogen, the United Kingdom).

Copolymerization of D,L-lactide and ε-caprolactone was performed in the presence of 0.05 mol % tin 2-ethylhexanoate. The ampule with the monomers was evacuated to 50–60 mmHg prior to sealing. The polymerization was performed at 150°C for 4 days (96 h). The synthesized polymer was dissolved in chloroform and precipitated into a tenfold volume of methanol. The copolymers were dried in air and then in a vacuum to constant weight and were stored in a desiccator over concentrated sulfuric acid. The yields and intrinsic viscosities of the reaction products are given in Table 1.

Films 65 μm thick were formed on a Teflon support by casting from a 10% solution of the copolymer in chloroform. After solvent evaporation, the films were dried in a vacuum to constant weight.

The structure of the copolymers was characterized by ^{1}H NMR (DPX 300 Bruker, 300 MHz) in $CDCl_3$.

The intrinsic viscosity of the copolymers was measured with an Ubbelohde capillary viscometer at 25°C in $CHCl_3$.

Degradation. Film specimens (15 × 15 mm, 0.1 g weight) were kept in PBS (pH 7.2) for 5, 14, and 28 days at 37°C. After that, the films were washed with distilled water and dried in air and then in a vacuum to constant weight, and the weight loss Δm and a decrease in the

intrinsic viscosity Δ[η] were measured:

$$\Delta m = \frac{m_0 - m_t}{m_0} \times 100\%,$$

where m_0 is the initial sample weight, and m_t is the sample weight after degradation by the time moment t;

$$\Delta[\eta] = \frac{[\eta]_0 - [\eta]_t}{[\eta]_0} \times 100\%,$$

where $[\eta]_0$ is the intrinsic viscosity of the initial polymer, and $[\eta]_t$ is that of the polymer after degradation by the time moment t.

Five specimens were taken for each point, and the measurement uncertainty was calculated.

Mechanical characteristics of polymer films. The mechanical properties were tested with an Instron 1122 universal testing machine in the active extension mode. The traverse velocity in the course of straining was 50 mm min^{-1}. All the tests were performed under ambient conditions (T = 20°C, 65% humidity). The thickness of each specimen was preliminarily measured with an electronic micrometer. The width of all the specimens being tested was 5 mm.

Isolation and cultivation of primary keratinocytes. The primary culture of human keratinocytes was obtained from the skin of healthy adult donors by cosmetic operation. Keratinocytes were isolated as follows [16]. The skin was washed with PBS, and after separation of the hypodermis, cut into small (5–10 mm) fragments, which were placed for 14–16 h at 4°C into PBS (pH 7.4) containing 0.2% collagenase and 0.5% dispase. After treatment with enzymes, the epidermis was separated and incubated in a solution containing 0.25% trypsin and 0.125% Versene for 7 min at 37°C. The enzyme was inactivated by adding 10% FBS and left for 20 min, after which the cells were precipitated by centrifugation. The precipitate was resuspended in a mixture of DMEM and F12 (3 : 1) with the addition of 10% fetal cow serum. The cell suspension was applied onto cover glasses coated with a film of the synthesized copolymers and cultivated for 2 days.

Evaluation of the state of keratinocytes on polymer films by fluorescence microscopy. After cultivation of keratinocytes on films for 1 or 2 days, the cultural medium was removed and the attached cells were washed with PBS. The cells were fixed with a 4% Formalin solution for 10 min and washed with three portions of PBS. Then, they were treated with 0.1% Triton X-100 for 15 min and, after similar washing with PBS, were stained for 10 min with rhodamine phalloidin and washed with PBS. Then, DAPI (Invitrogen, the United Kingdom) was applied for 5 min. After the subsequent washing, the specimens were placed into the embedding medium. The actinic structures of cells were analyzed with a confocal microscope (LSM 5 Pascal, Germany). Qualitative evaluation of the attached and spread cells was made using ImageJ program [17].

RESULTS AND DISCUSSION

The copolymers were synthesized by bulk polymerization of a mixture of ε-caprolactone and D,L-lactide in the presence of tin 2-ethylhexanoate at 150°C in an evacuated ampule. The molar ratio of the monomer mixture and initiator was 2000 : 1. The reaction follows the scheme shown below.

Characteristics of polymers. The structure of the synthesized copolymers was studied by ^{1}H NMR spectroscopy in $CDCl_3$.

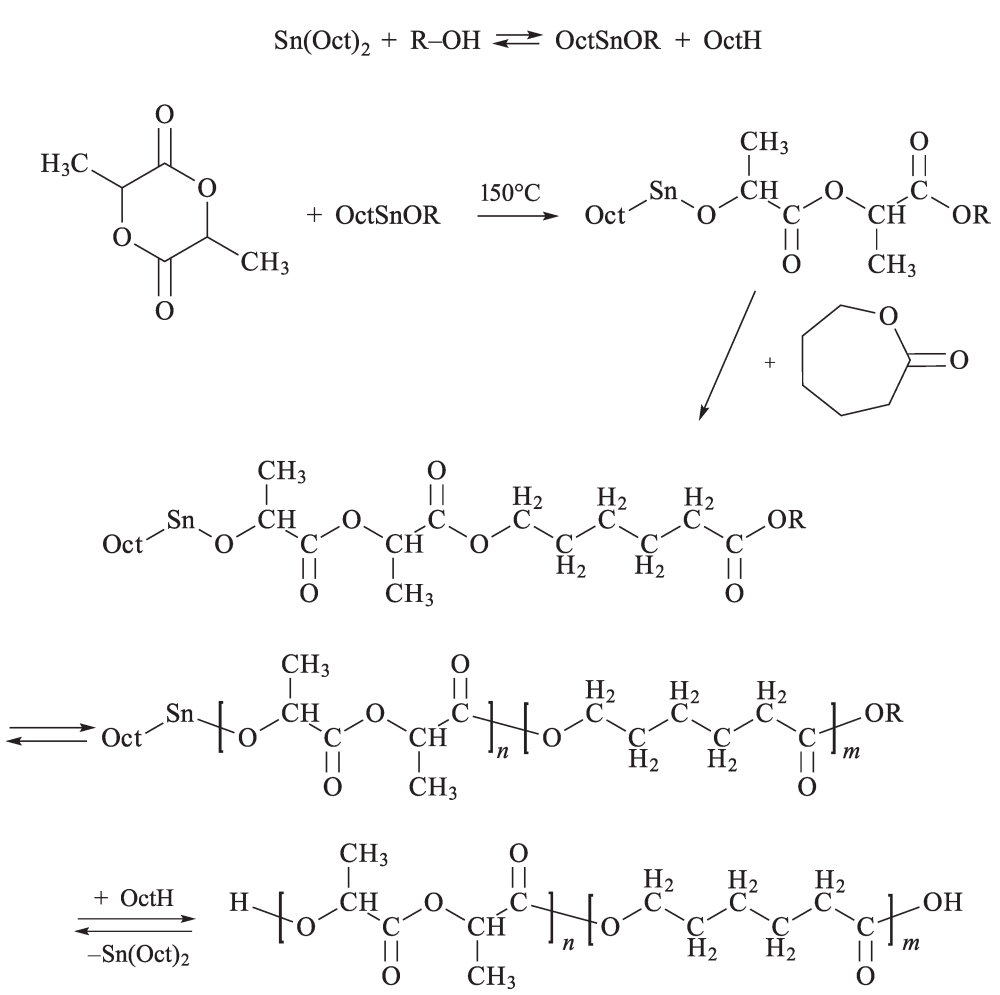
The signal at δ = 5.2 ppm corresponds to the methine protons of lactide units, and the signals at δ 2.3 and 4.1 ppm, to methylene protons of caprolactone units. The signal intensities in the ^{1}H NMR spectra are given in Table 2. The copolymer composition and comonomer ratio were estimated from the ^{1}H NMR data.

According to published data, the reactivity of D,L-lactide is higher than that of ε-caprolactone [13]. However, the NMR data show that both monomers enter into the reaction and that their ratio in the copolymer is close to the initial ratio of the monomers in the reaction mixture (Table 1). These results are due to the fact that the polymerization of D,L-lactide is reversible and, when the equilibrium is attained, the ε-caprolactone present in the mixture enters into the reaction [18, 19]. This mechanism of the copolymerization of D,L-lactide and ε-caprolactone ensures uniform distribution of comonomer units in the chain.

Data on the intrinsic viscosity and yield of the polymers are given in Table 1. As can be seen, an increase in the caprolactone content in the initial monomer mixture leads to a decrease in the intrinsic viscosity and, correspondingly, to a decrease in the molecular mass of the copolymers.

Degradation. Degradation of the copolymers in vitro was studied in 0.10 M PBS (pH 7.2) at 37°C from the variation of the intrinsic viscosity and weight loss of

Scheme. Copolymerization.



the polymer films. The intrinsic viscosity as a function of time is plotted in Fig. 1.

As seen from the figure, in all the cases the intrinsic viscosity decreases with time. The degradation rate decreases in proportion with an increase in the caprolactone content of the copolymer. The intrinsic viscosity changed to the least extent for the samples containing 15 and 20% ε-caprolactone units. The D,L-lactide homopolymer degrades faster than the copolymers do. This trend is due to specific structural features of the homopolymer and copolymers. Namely, introduction of aliphatic ε-caprolactone units into the polymer chain leads to a decrease in the copolymer hydrophilicity and hence in the degradation rate. Similar trends were repeatedly demonstrated in other studies concerning D,L-lactide–ε-caprolactone copolymers. The presence of the lactide in the polymer chain always leads to a decrease in the copolymer degradation rate [20].

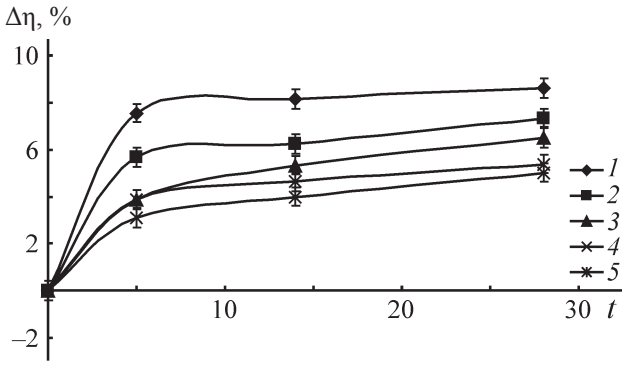


Fig. 1. Decrease in the intrinsic viscosity of the polymers, Δη, as a function of degradation time *t*. (*1*) PL; CL content, %: (*2*) 5, (*3*) 10, (*4*) 15, and (*5*) 20; the same for Fig. 2.

The variation of the film weight with time is shown in Fig. 2. The most hydrophilic sample, D,L-lactide homopolymer, loses the weight to the greater extent than the copolymers do. Analysis of the intensity of

Table 2. Signal intensities in ^{1}H NMR spectra

Sample no.	Intensity of signal at indicated δ, ppm				
	1.3–1.4	1.6	2.3	4.0–4.2	5.1–5.3
1 (5 mol % CL)	0.12	3.15	0.077	0.072	1
2 (10 mol % CL)	0.28	3.43	0.25	0.22	1
3 (15 mol % CL)	0.36	3.74	0.34	0.33	1
4 (20 mol % CL)	0.45	3.85	0.44	0.43	1

a decrease in the weight and intrinsic viscosity of the samples after incubation under physiological conditions shows that initially the weight loss was less intense than a decrease in the intrinsic viscosity.

Determination of the strain and strength characteristics. The extension diagrams of film specimens prepared from polylactide and copolymers with 5, 10, and 15% ε-caprolactone units are shown in Fig. 3. Measurements were performed with film specimens of 5 × 40 mm size and 50 μm thickness.

The stress and strain characteristics of all the samples, obtained from the extension diagrams, are given in Table 3.

An increase in the content of ε-caprolactone units leads to significant changes in the mechanical properties of the copolymer films.

In the extension diagrams of the films prepared from the homopolymer and copolymer with 5% ε-caprolactone content, there is a well-defined maximum indicative of the existence of the forced elasticity limit. All the specimens break at 11–12% strain and the stress from 0.87 to 9.9 MPa. This breaking strain shows that the forced rubber-like elastic strain mode is not realized to a full measure; in our case, this may be due to the specimen shape (thin films) and to low molecular masses of the polymers.

In the extension diagrams of the copolymer films with 10 and 15% content of ε-caprolactone units, there are no zones of elastic and forced elastic strain. Such shape of the extension diagrams can correspond to the strain of an amorphous polymer in the hyperelastic state. It is also seen from the results obtained that, as the ε-caprolactone content of the copolymer is increased, the stresses tend to decrease throughout the range of extension strains. The copolymer composition exerts the strongest influence on the elastic modulus of the films: The initial modulus of the copolymer film with 15%

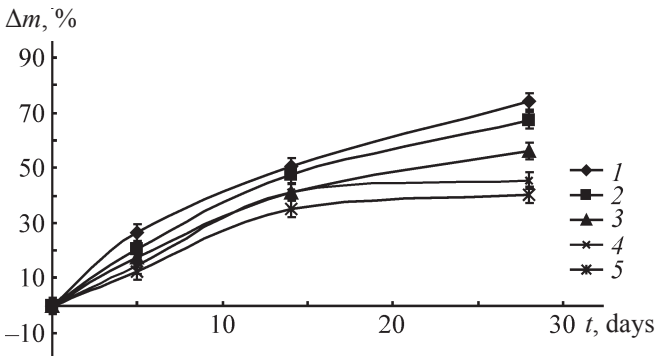


Fig. 2. Film weight loss Δ*m* as a function of degradation time *t*.

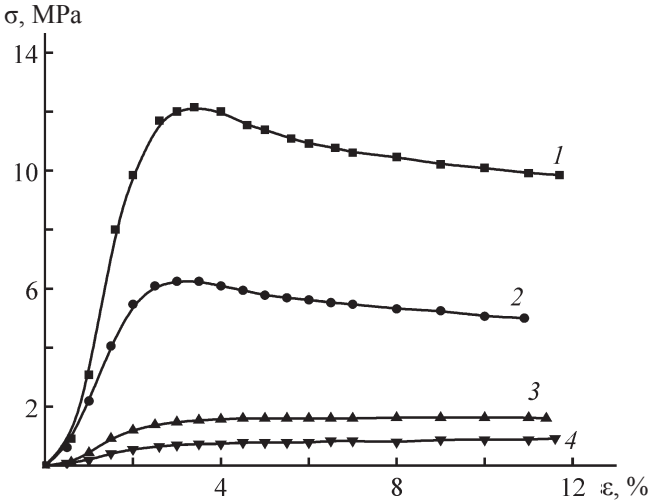


Fig. 3. Extension diagrams of films of polylactide and D,L-lactide–ε-caprolactone copolymers containing 5, 10, and 15% ε-caprolactone. (σ) Stress and (ε) relative strain. (*1*) PL; CL content, %: (*2*) 5, (*3*) 10, and (*4*) 15.

Table 3. Strain and strength characteristics of polylactide and D,L-lactide–ε-caprolactone films

Sample	Elastic modulus E, MPa	Elongation in yield point, ε_y, %	Stress in yield point, σ_y, MPa	Elongation at break, ε_b, %	Breaking stress, σ, MPa
Polylactide, 0% CL	820	3.2	12	11.7	9.9
5% CL	512	2.7	6.2	11	4.9
10% CL	98	2.3	1.4	11.4	1.6
15% CL	46	2.3	0.65	11.6	0.87

content of ε-caprolactone units is 18 times lower than that of polylactide. A significant factor is that the material pliability for films with 10 and 15% ε-caprolactone content is uniform throughout the extension range. The tensile elastic modulus correlates with the strain characteristics of the material under the action of bending loads. As noted above, increased rigidity of the films, preventing their uniform arrangement together with the cells on a wound bed, is a significant drawback of this material. Introduction of ε-caprolactone units allows preparation of materials that are considerably more pliable under the action of bending strain compared to the pure polylactide. Specifically this property allows uniform distribution of films of this material on a wound bed, thus considerably enhancing the efficiency of the regenerative process.

Thus, the results of studying the degradation of films under physiological conditions and the strain and strength characteristics of the films allowed us to choose films of D,L-lactide–ε-caprolactone copolymer with 15% ε-caprolactone content for cultivation of human keratinocytes with the aim of their subsequent transplantation on the wound bed. The films of this copolymer significantly surpass in the deformability the films of poly(D,L-lactide) or of the 95/5 or 90/10 copolymer. Increased deformability considerably improves the operation characteristics of the films in their transplantation together with cells onto a wound. It should also be noted that introduction of 15% ε-caprolactone into the copolymer decreases the film degradation rate under physiological conditions; changes in the weight and intrinsic viscosity of the copolymer are less pronounced compared to poly (D,L-lactide).

To evaluate the effect of the copolymer composition on the amount of the attached keratinocytes and on their spreading, the cells were cultivated on the prepared films for 2 days.

The keratinocyte cultivation was monitored using cover glasses coated with a 0.1 mg mL^{-1} solution of type I collagen (standard cultivation conditions). Films of poly(D,L-lactide) were used as an additional reference for comparison. After cultivation for 1 and 2 days, keratinocytes were fixed and stained with rhodamine phalloidin to reveal actinic structures and with DAPI to reveal nuclei. The quantitative evaluation of the degree of attachment and spreading of the cells on the films was evaluated using ImageJ program (Fig. 4).

As seen from Fig. 4, after cultivation for 1 day the amount of attached cells on poly(D,L-lactide) films is no lower than that on the cover glass coated with a collagen solution (control), whereas the amount of attached cells on films of the 85 : 15 copolymer is lower (Fig. 4a). However, the degree of keratinocyte spreading on the copolymer films is considerably higher than that on poly(D,L-lactide) (Fig. 4c) and similar to that on the collagen-coated surface. After 2 days, virtually all the keratinocytes became well spread on the copolymer film and on the cover glass with collagen, whereas on poly(D,L-lactide) the cells remained unspread (Fig. 4d). Figure 4 also shows that, after 2-day cultivation, the amount of keratinocytes attached to the polymer surfaces in both cases was lower than the amount of the cells attached to the collagen-coated surface (Fig. 4b). These results are in good agreement with our previous data and with the data of other authors [8, 21]. On the other hand, the ability of keratinocytes for efficient spreading on films of D,L-lactide–ε-caprolactone copolymer compared to the collagen-coated surface (Fig. 4c) already after 1-day cultivation requires further detailed study.

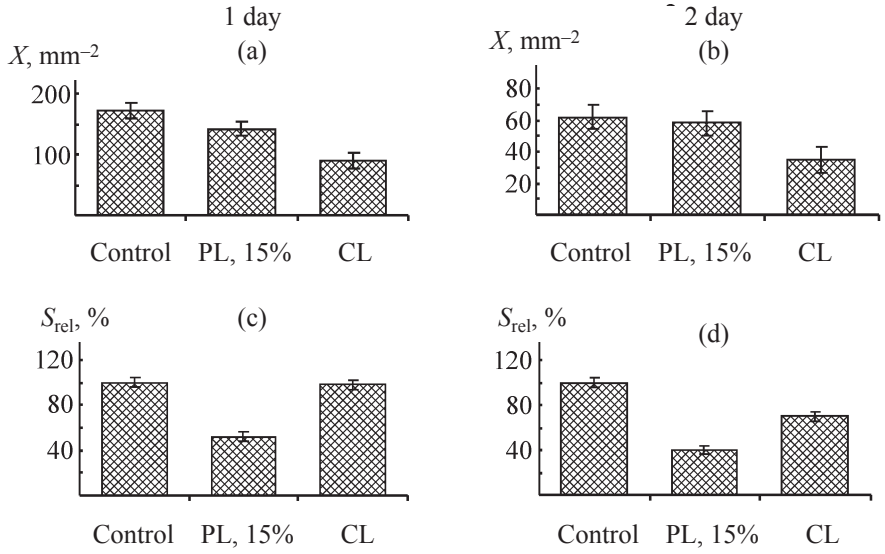


Fig. 4. Diagrams illustrating the attachment and spreading of keratinocytes on polymer films. (X) Number of cells per mm^2 and (S_{rel}) relative area occupied by the cells. (a, b) Number of cells attached to polymer films and (c, d) extent of spreading on polymer films. Cultivation time, days: (a, c) 1 and (b, d) 2.

CONCLUSIONS

(1) The film degradation rate decreases in proportion with an increase in the caprolactone content of the copolymer. The intrinsic viscosity decreased to the least extent for the samples with 15 and 20% ε-caprolactone units.

(2) In the entire range of tensile strains, the stress tends to decrease with increasing ε-caprolactone content of the samples. The ε-caprolactone content of the copolymer influences the elastic modulus of the films.

(3) The 85/15 copolymer films are inferior in the amount of attached cells only to the poly(D,L-lactide) films. The area occupied by the cells is larger on the 85/15 copolymer films compared to the poly(D,L-lactide) films.

(4) The results of studying the degradation and strain and strength characteristics of the films show that the films of the copolymer with 15 mol % ε-caprolactone are the most suitable for keratinocyte cultivation.

(5) The films obtained show promise for transplantation of keratinocytes with the aim of regeneration of damaged skin.

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