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DIFFERENT SOURCE ATELOCOLLAGEN THIN FILMS: PREPARATION, PROCESS OPTIMISATION AND ITS INFLUENCE ON THE INTERACTION WITH EUKARYOTIC CELLS

RAZLIČEN IZVIR ATELOKOLAGENSKIH TANKIH PLASTI: PRIPRAVA, OPTIMIZACIJA PROCESA IN VPLIV NA INTERAKCIJE Z EVKARIONTIČNIMI CELICAMI

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Collagen thin films were prepared via bovine atelocollagen matrices. The film casting was carried out by using different culture dishes, concentrations, equipment, drying processes and periods of time. In order to optimise the repeatability and reproducibility, microscopic analyses were utilised to explore the film quality and topographical patterning. In addition, the human immortalised non-tumorigenic keratinocyte cell line (HaCaT) was seeded onto the obtained specimens, and the cell proliferation was determined by using the MTT assay. These results indicated how the substrate, its concentration and processing conditions influence the cellular response. The attempted technique shows itself to be an excellent procedure for continuous collagen film preparation with optimal cell-proliferation rates, which may potentially be used in tissue engineering or wound-healing applications.

Keywords: atelocollagen thin films, film optimisation, film quality, surface topography, eukaryotic cell response

Kolagenska tanka plast je bila narejena z govejo atelokolagensko matriko. Priprava plasti je potekala na različnih gojiščih, pri različnih koncentracijah, opremi, postopkih sušenja in pri različnih časih. Za doseganje optimalne ponovljivosti in obnovljivosti so bile uporabljene mikroskopske analize, s katerimi je bila ugotovljena kakovost plasti in njihov topografski vzorec. Poleg tega so bile na vzorce nanesene imortalizirane nekancerogene celice linije keratinocita (HaCaT), njihova proliferacija pa je bila ugotovljena z MTT-preizkusom. Rezultati prikazujejo, kako podlaga, njena koncentracija ter procesne razmere vplivajo na biološki odziv. Tovrstna tehnika je edinstven postopek za izdelavo kontinuirnih kolagenskih plasti za optimalno proliferacijo celic, ki je potencialno uporabna v tkivnem inženirstvu ali za celjenje ran.

Ključne besede: atelokolagenske tanke plasti, optimizacija plasti, kvaliteta plasti, topografija površine, odziv evkariontičnih celic

1 INTRODUCTION

Collagen is a fibrous protein that is present in nearly all mammalian tissues. It constitutes approximately 25 % of the whole-body protein content[1] and its abundance is mainly centred on connective tissues, such as tendons, ligaments and cartilage. Skin also contains this protein, which is involved in prime biological functions, such as tissue formation, cell attachment and proliferation.[2] Around 19 proteins are classified as collagen. Moreover, there are several proteins that have collagen domains.[3] Although the 20 standard amino acids are encoded in collagen biosynthesis, the general sequence is $(X\text{-}Y\text{-}Glycine)_n$, where proline is recurrently in the X-position, and 4-hydroxyproline, which is almost unique to collagen, in the Y-position of the sequence (**Figure 1a**).[4,5]

This protein is made up of three polypeptide strands, each chain is a left-handed helix, and the three chains are coiled around each other in a right-handed super-helix.[6,7] As a biomaterial for industrial applications, collagen has been widely used in many fields, such as cell cultures, cosmetic and food products and medical devices.[8]

As for medical applications, this protein is considered as a primary source in biomedical applications and one of the most useful biomaterials because of its excellent biocompatibility, immunogenicity and biodegradability.[9–11] Unfortunately, native collagen has some difficulties with its tractability. For example, it may not be processed by injection moulding or any conventional extrusion technique.[12]

Collagen treated either by enzymatic digestion or by salt/acid extraction is so-called atelocollagen. This treatment leads towards crosslinks breaking amongst the collagen molecules, resulting in soluble triple helices lacking of telopeptides, which has the same physical properties as untreated collagen (**Figure 1b**).[13]

Atelocollagen possesses enormous assets; for instance, it is soluble in an acid pH, and its liquid form is tractable and free of telopeptides, which guaranties a low immunogenicity. Thus, it is employed for a wide range of purposes, including wound healing, vessel prostheses, haemostatic agent and tissue engineering.[14–18]

Despite the plethora of collagen-related publications, and to the best of our knowledge, there is no general

description of the preparation of atelocollagen thin films. Therefore, the aim of this contribution is the preparation and optimisation of collagen thin films. These findings seek to enlighten some of the issues involved in collagen sheet forming, film casting and the use of atelocollagen as a biomaterial.

2 PREPARATION OF ATELOCOLLAGEN FILMS

Selecting a proper collagen source is the most important issue before preparing collagen films. Four sorts of atelocollagen were employed across the trial, i.e., an emulsion of atelocollagen from bovine Achilles tendon, which contents 1.4 % of atelocollagen with a pH of 3.5; a gel of atelocollagen from bovine splits, 16.2 % of atelocollagen, pH 5.16 (Splits 1); a gel of atelocollagen from bovine splits, 11.8 % of atelocollagen, pH 3.7 (Splits 2) and an emulsion of atelocollagen from bovine tendons, 3.6 % of atelocollagen, pH 3.3. These starting materials were provided by Vipo A. S., and all of them were produced by trypsin enzymatic digestion. Three main factors were taken into account: atelocollagen concentration, processing factors such as mixing and casting solution volume. Each atelocollagen source was solubilised in a aqueous acetic acid solution 0.1M to finally obtain the mass fractions (0.5, 0.25, 0.1 and 0.05) %. A solution of acetic acid 0.1M was used as a solvent throughout this study; on account of it being recommended and employed in previous researches.[19–21] It is because pH values within 1.0 and 4.0 have the best collagen-solubility yields.[22,23] The measured pH of the experimental solution was between 3.5 and 4.0.

The tested samples were homogenised and casted in culture dishes by using stirring machines with distinct rotational frequencies and pouring volumes. An IKA RCT stirring machine (IKA® works, Inc, Germany) and Merci 1500 (Merci S. R. O, Czech Republic) were used for the experiments.

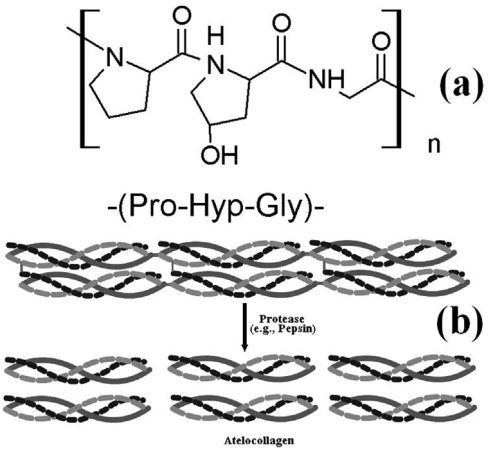

Figure 1: a) Collagen typical primary sequence; b) atelocollagen via enzymatic digestion

Slika 1: a) Značilno primarno zaporedje kolagena; b) atelokolagen z encimsko razgradnjo

A rotational frequency of 1000 r/min was set as the appropriate one, since this speed provided an efficient flock reduction. Higher frequencies may cause sample spilling, whereas lower frequencies are insufficient for the appropriate preparation of films within a reasonable processing time. The mixture is referred as the state formed by a complex of two or more ingredients, whilst mixing is an operation intended to reduce the non-uniformity of a mixture and it may be achieved by inducing the physical motion of the ingredients. The mixing time was a crucial factor; ensuring 4 h of mixing, the solution was homogeneous and without atelocollagen flocks. After an hour of mixing, medium-size flocks were visible in the solution, and those were progressively reducing their sizes. Nevertheless, the turbidimetry assay that measures transmitted light, and is directly proportional to the concentration and depth of the dispersion, indicates that approximately one hour is enough for serving, since the transmittance started to be constant (**Figure 2**).

This information may be corroborated either with Scanning Electron Microscopy (SEM) images, which illustrate the relation between surface-quality and mixing time (**Figure 3**), or in **Table 1**, which lists the percentage of transmittance before beginning the enquiry.

Table 1: Mass fractions of transmittance with respect to concentration (*w*/%)

Tabela 1: Delež prepustnosti glede na koncentracijo v masni deležih (*w*/%)

Source	0.5 %	0.25 %	0.1 %	0.05 %
Atelocollagen from bovine splits 1	80.1	73.4	51.2	28.5
Atelocollagen from bovine splits 2	91.2	73.6	56.5	32.0
Atelocollagen from bovine tendons	87.7	72.7	50.4	27.8

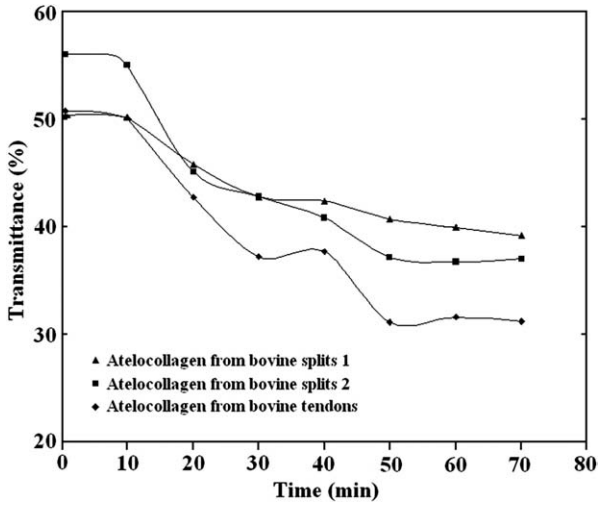

Figure 2: Turbidimetry assay of different atelocollagen solutions, studied concentration 0.1 %

Slika 2: Preizkus turbidimetrije različnih atelokolagenskih raztopin; raziskovana koncentracija 0,1 %

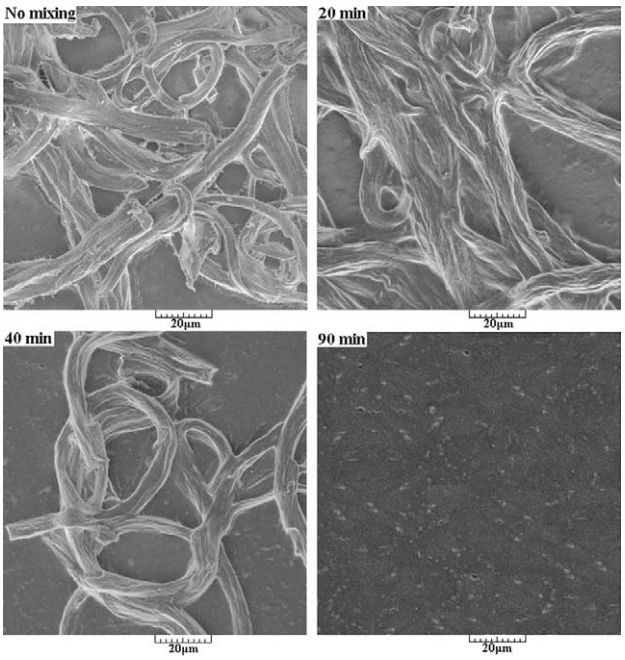

Figure 3: Mixing progress: emulsion 0.1 % of atelocollagen from bovine tendons. SEM images taken at different mixing times.
Slika 3: Proces mešanja: emulzija 0,1 % atelokolagena iz goveje kite. SEM-posnetki po različnih časih mešanja.

The turbidimetry assay was carried out on a Helios γ-spectrophotometer (Thermo scientific, USA) at 422 nm.

All the atelocollagen solutions were disposed into the culture dishes (TPP, Switzerland) 30 min after mixing, to avoid bubbles coming from the solution stirring, which alter surface texture. When the solutions were casted immediately, the subsequent films had a rough appearance, along with collagen congregates, which indeed deteriorate the surface quality (**Figure 4a**).

The employed culture dishes were of (9.0, 6.0 and 4.0) cm diameter. The first attempt involved the 9.0 cm ones and volumes of (30, 20, 10 and 5) mL of the solution were casted into. The casted films were kept standing at lab temperature (20–24 °C) for three days to evaporate the excess solvent.

Casted solutions of 20 mL and 30 mL are also suitable for making collagen films; nonetheless, those volumes need more time for optimal solvent evaporation. If 10 mL is casted, its films get their shapes in two days, but the solvent still remains (eye observation and sensory evaluation). The extra day is for completing the solvent evaporation.

A total of 5 mL was discarded as a pouring volume, because it scarcely took the shape of the 9.0 cm Petri dishes. Then, it was decided that 10 mL should be the starting volume for the culture dishes of this diameter.

The vacuum and the temperature may be utilised for film drying, thereby reducing the evaporation time. However, fast solvent evaporation and vacuum influence the surface smoothness (**Figures 4b** and **4c**).

With reference to temperature, it may not exceed 30–35°C, which is on average the denaturation temperature of collagen acid solutions, and refers to the collapse of the collagen triple helix to a random coil configuration.[24–27] As the vapour pressure rises, the surface roughness increases, and to render smooth films, it is extremely important to focus on the employed solvent and its evaporation. Solvents with low evaporation rates tend to produce better quality films than fast evaporating ones.[28,29]

3 SURFACE MORPHOLOGY

The specimens made from 10 mL had the best film appearance (dry, smooth, resilient and the solvent seemed to be thoroughly evaporated). For example, the digital camera images depict a surface that apparently has neither any eye-visible damage nor scratches (**Figure 5**). This assessment is reinforced by the SEM images, where the surfaces are relatively smooth, except for some

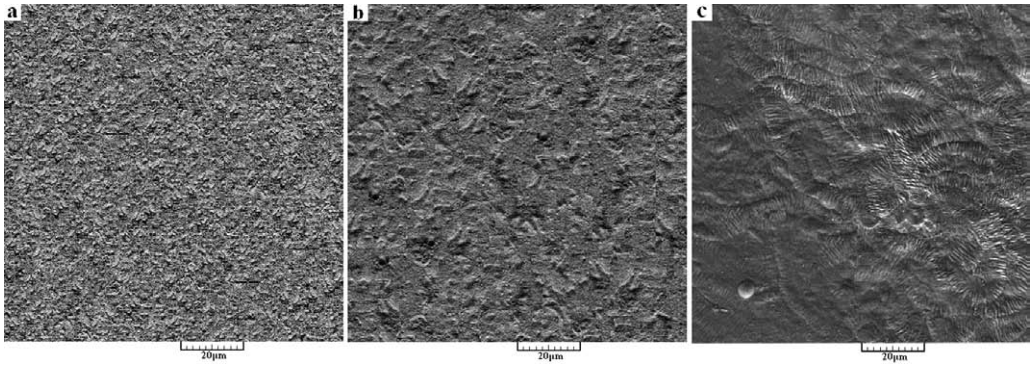

Figure 4: a) Atelocollagen film obtained from a solution of 0.1 % of the mass fraction atelocollagen from bovine tendons that was poured immediately after mixing; b) atelocollagen films cast and dried at 30 °C of 0.1 % atelocollagen from bovine tendons; c) by using a desiccator of 0.1 % atelocollagen from bovine splits 1
Slika 4: a) Atelokolagenske plasti, pridobljene iz 0,1 % masnega deleža raztopine atelokolagena iz goveje kite, ki je bil nanesen takoj po mešanju; b) ulita in sušena atelokolagenska plast pri 30 °C iz 0,1 % atelokolagena iz goveje kite; c) z uporabo eksikatorja iz 0,1 % atelokolagena iz govejega dela 1

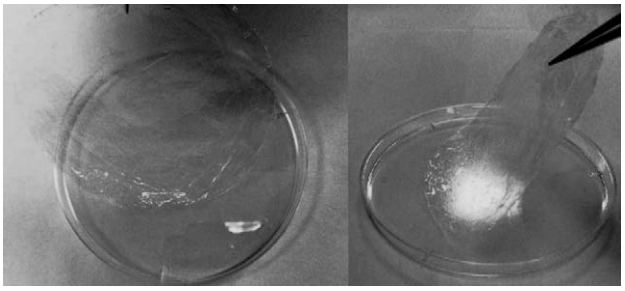

Figure 5: Glimpse at the thin films of 0.1 % of the mass fraction of atelocollagen from bovine splits 2 by a standard camera with the corresponding brightness and contrast adjustments
Slika 5: Pogled na tanko plast 0,1 % masnega deleža atelokolagena iz govejega dela 2 s standardno kamero s primerno nastavljeno svetlostjo in kontrastom

wavy areas, which may result either from small collagen flocks or a product of solvent evaporation (**Figure 6**).

Scanning electron microscopy (SEM) was performed in a VEGA II LMU microscope (Tescan s. r. o, Czech Republic) operated in high vacuum/secondary electron imaging mode at an accelerating voltage of 5 kV. The specimens were coated with a thin layer of gold/palladium alloy and tilted at 30° to attain a better observation of the surface topography. The images were taken at a magnification of 2000-times.

4 THIN-FILM NORMALISATION

To normalise the procedure for multiple diameters, the height of served solutions in the Petri dishes was ascertained by means of an equation that signifies the

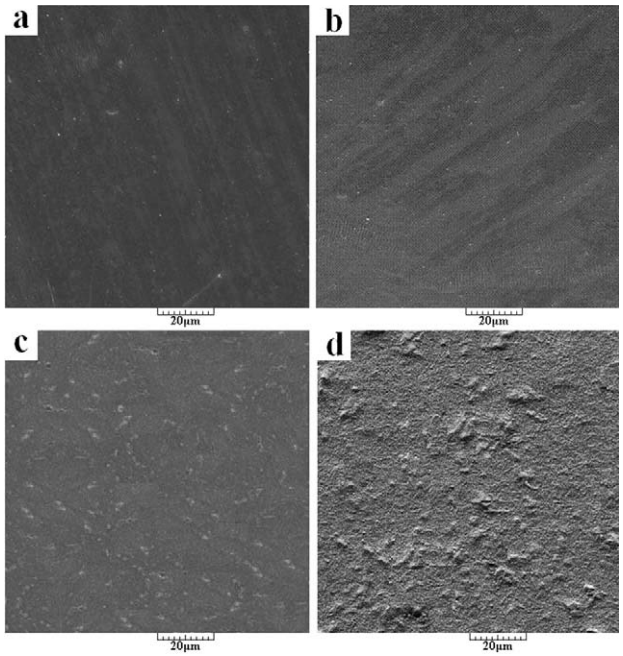

Figure 6: Surface morphology of the atelocollagen thin films from: a) bovine splits 1; b) bovine splits 2; c) bovine tendons; d) bovine tendons
Slika 6: Morfologija površine atelokolagenske plasti iz: a) govejega dela 1; b) govejega dela 2; c) goveje kite; d) goveje kite

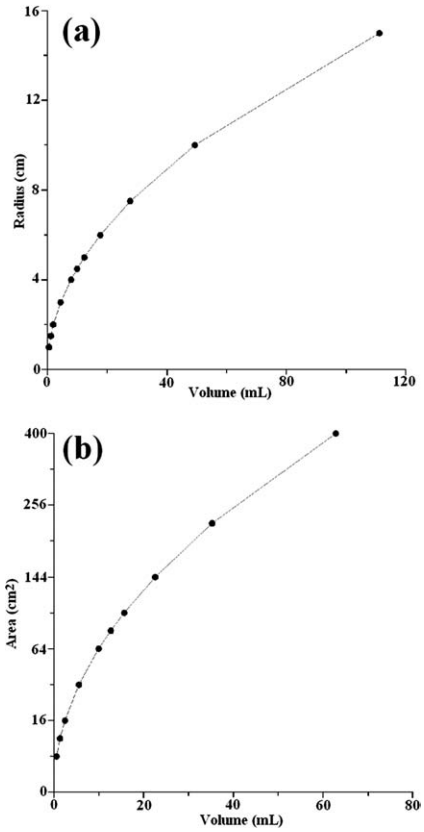

Figure 7: a) Model for Petri dishes; b) model for rectangular cuboids sheets
Slika 7: a) Model za petrijevke; b) model za pravokotne kubične liste

volume of a cylinder $v = \pi r^2 h$, where v is the volume, r is the radius and h is the height. The idea was to keep the same height no matter the diameter (twice the radius) of the culture dishes, *i.e.,* same height, distinct radii and thus, different volumes. The height was calculated by using 10 cm^3 as an initial volume. As was mentioned, 10 mL (equivalent to 10 cm^3) was the selected volume for the 9.0 cm diameter. The obtained volumes from the previously calculated height were 4.5 mL and 2.0 mL for the 6.0 cm and 4.0 cm diameters, respectively.

In order to get the same film thickness, the height has to be settled as a constant. The model may be applied to any Petri dish, as it is described in **Figure 7a** or numerous forms that have a known area. **Figure 7b** shows the model that may be used for rectangular sheets. In any rectangular cuboid form, the volume is defined as $v = lwh$, where l is the length, w is the width and h is the height. If the height (film thickness) remains steady and the length and width (area) are known, the volume may be ascertained.

This trial was conducted by following two fundamental concepts: repeatability and reproducibility. The former is the effort to keep constant conditions by using the same instruments in a short period of time; and the latter is the one with different instruments and other periods of time.

5 CELL RESPONSE

A human immortalised non-tumorigenic keratinocyte cell line was supplied by Cell Lines Service (Catalogue No. 300493, Germany). Dulbecco's modified eagle medium-high glucose, supplemented with 10 % foetal bovine serum and Penicillin/Streptomycin, 100 U/mL (100 µg/mL) respectively (PAA Laboratories GmbH, Austria) was used as a culture medium.[30] HaCaT cells in the exponential growth phase were seeded onto the thin films at a concentration of cells 1×10^5 mL^{-1} and incubated at 37 °C with 5 % CO_2 in humidified air.

The cell proliferation was determined after 4 d in the culture with the MTT cell proliferation assay kit (Invitrogen Corporation, USA). The formazan concentration was measured in a Sunrise microplate absorbance reader (Tecan, Switzerland) at 570 nm. The photomicrographs were taken by using an inverted phase-contrast microscope Olympus CKX41 (Olympus, Germany) with an optical zoom of 40-times.

The HaCaT keratinocyte cells behaviour on the prepared films was evaluated with the MTT assay and the results are given in **Figure 8**. It was found that after four days of cultivation, in terms of cost-effectiveness the films made from 0.1 % were the most suitable. Regardless of the atelocollagen matrix, the cell proliferation on the films prepared at this concentration exhibits the highest rate. In contrast, higher concentrations of collagen (0.5 % and 0.25 %) seem to be detrimental to the keratinocytes cells.

As far as surface morphology is concerned, the films that were dried at 30 °C, (with faster solvent evaporation and rougher surfaces than the lab temperature ones) also had thriving cell proliferation rates (**Figure 8g**). Consequently, film drying at this temperature may be used without affecting atelocollagen-scaffold quality at all. It was previously demonstrated that the keratinocyte cells adhere and proliferate satisfactorily on both smooth and rough surfaces, and also the pivotal role of chemistry and topography as cell regulatory factors.[31–33]

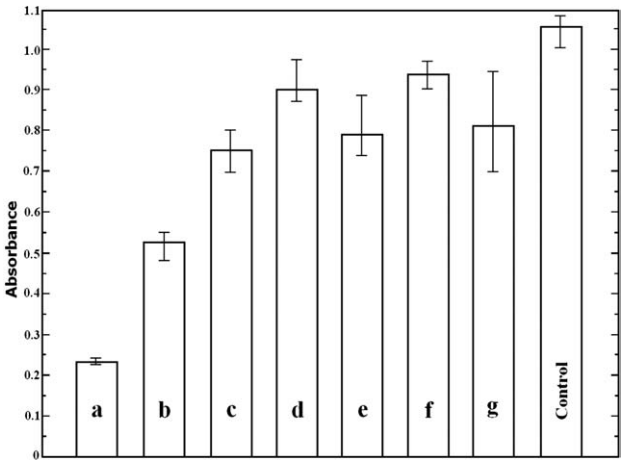

Figure 8: Comparison of HaCaT cell growth measured by MTT assay at 570 nm on: a) 0.5 %; b) 0.25 %; c) 0.05 %; d), e) and f) 0.1 % of the mass fraction of atelocollagen from bovine tendons, atelocollagen from bovine splits 1 and atelocollagen from bovine splits 2 respectively; g) 0.1 % film, dried at 30 °C. Error bars signify standard deviations.

Slika 8: Primerjava rasti celic HaCaT, izmerjene z MTT-preizkusom pri 570 nm: a) 0,5 %; b) 0,25 %; c) 0,05 %; d), e) in f) 0,1 % masnih deležev atelokolagena iz goveje kite, atelokolagena iz govejega dela 1 in atelokolagena iz govejega dela 2; g) 0,1-odstotna plast, sušena pri 30 °C. Odmiki na grafu so prikaz standardne deviacije.

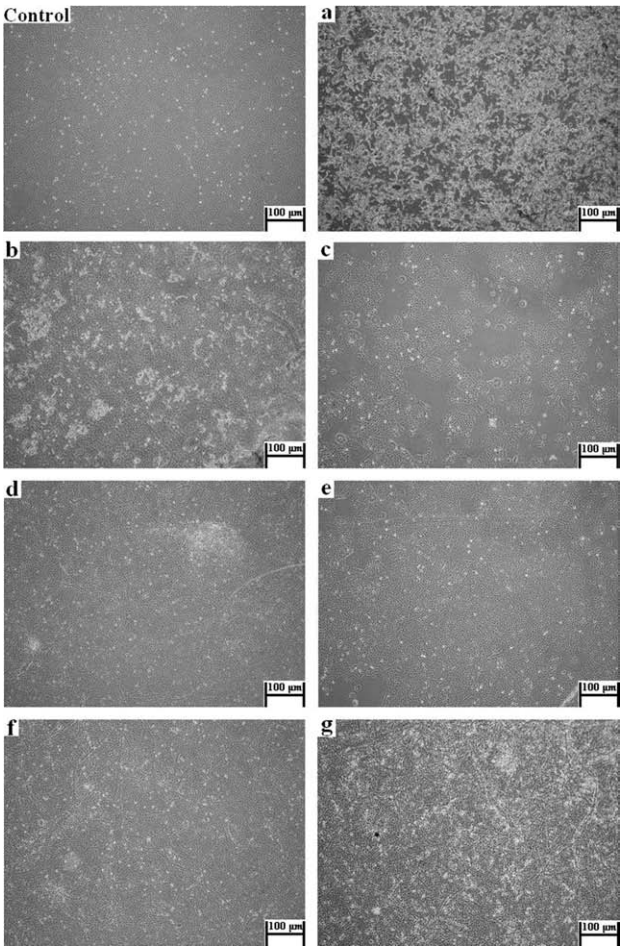

Figure 9: Photomicrographs of human skin HaCaT keratinocytes in culture upon collagen compared with control: a) 0.5 %; b) 0.25 %; c) 0.05 %; d), e) and f) 0.1 % of the mass fraction of atelocollagen from bovine tendons, atelocollagen from bovine splits 1 and atelocollagen from bovine splits 2; g) 0.1 % film, dried at 30 °C

Slika 9: Slike keratinocitov iz človeške kože HaCaT v kulturi s kolagenom v primerjavi s kontrolo: a) 0,5 %; b) 0,25 %; c) 0,05 %; d), e) in f) 0,1 % masnih deležev atelokolagena iz govejega kite, atelokolagena iz govejega dela 1 in atelokolagena iz govejega dela 2; g) 0,1-odstotna plast, sušena pri 30 °C

This outcome was also qualitatively appraised by the photomicrographs in **Figure 9**, which show the cell aggregates that are adhered on the film surfaces. It may be seen that the film obtained from 0.5 % has a vast amount of collagen flocks, which presumably hinder the HaCaT cell growth.

Nevertheless, the mechanisms of keratinocytes adhesion and proliferation are still unclear, even though it is well known that hydrophilic surfaces (like these ones) are favourable for HaCaT cell growth.[34–36] For this reason, a protocol for continuous and effective substrate

preparation is of paramount importance. Atelocollagen is certainly a potential scaffold for cell growth purposes, which also has the advantage of being eliminated by degradation processes similar to the metabolism of endogenous collagen.[37]

6 CONCLUSIONS

In this contribution, the adopted technique for atelocollagen thin-film preparation was confirmed to be a cost-efficient procedure, independent of the equipment or the time. It increases in importance due to the difficulties that collagen has for its manipulation. The materials referred to herein as 'atelocollagen films' were succinctly characterised by microscopic analysis. In addition, HaCaT keratinocytes cell adhesion was successfully accomplished. This effort may be adjusted to diverse atelocollagen matrices. Hence, the present approach strengthens the knowledge in the use of atelocollagen as a prospective scaffold, and indeed in the development of suitable materials for tissue-engineering and wound-healing applications.

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