# ARTIFICIAL EXTRACELLULAR MATRICES OF COLLAGEN FIBRILS AND LACTOFERRIN AS COATINGS TO ENHANCE OSTEOBLAST BEHAVIOR

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## Abstract

*Lactoferrin, a glycoprotein found in milk, has stimulated osteoblast proliferation and differentiation, but has remained relatively unexplored as a biomaterial component. In this study, artificial extracellular matrices consisting of fibrils of collagen type I containing lactoferrin were used as coatings for the biocompatible polymer poly(lactic-co-glycolic acid) (PLGA).*

*The numbers of cells, their viability and proliferation rate were evaluated in various time intervals. Additionally, cell initial spreading area on day 1 was measured.*

*The results show that lactoferrin accelerates fibrillogenesis, leads to increased osteoblast cell numbers 1 and 3 days post-seeding, and encourages their proliferation in each of the tested time intervals.*

*Keywords: coating, collagen, osteoblast, proliferation, extracellular matrix, lactoferrin*

## Introduction

Lactoferrin is an 80 kD glycoprotein found in milk, which has stimulated osteoblast proliferation and differentiation as a component of a cell culture medium [1,2]. As a component of collagen membranes, lactoferrin has also promoted osteoblast differentiation [3]. However, collagen-lactoferrin interactions remain relatively unelucidated in the literature. In addition, the effect of lactoferrin as a biomaterial coating is relatively unexplored. In this study, artificial extracellular matrices consisting of collagen type I fibrils were used to coat sheets of the widely-used biocompatible polymer material poly(lactic-co-glycolic acid) (PLGA), as in our previous work [4]. Lactoferrin was incorporated into fibrils during fibrillogenesis, or during fibril formation. PLGA was coated by allowing these fibrils to adsorb onto the PLGA surface. Collagen gels formed as a result of fibrillogenesis. The effect of lactoferrin on the kinetics of fibrillogenesis was also investigated.

## Materials and methods

Collagen fibril gels 0.5 mL in volume were formed according to the Karamichos method [5]. Briefly, 0.4 mL of type I rat tail collagen (BD Biosciences, 354231) at a concentration of 1 mg/mL in 0.1 M HCl was added to 0.05 mL of 10 x Eagle's Minimum Essential Medium (MEM; Sigma-Aldrich, M0275) and either 0.05 mL 10 mg/ml lactoferrin (from bovine milk;

Sigma-Aldrich, L9507) in Milli-Q water or 0.05 mL Milli-Q. This yielded a final collagen concentration of 0.8 mg/mL. The lactoferrin concentrations were 1 mg/mL or 0 mg/mL for the lactoferrin-free controls. After dropwise neutralization with 1 M sodium hydroxide, gel formation took place at 37°C in a water bath.

The gelation kinetics at 37°C was investigated by performing rheometry using a flat plate-shaped rotating head of diameter 25 mm and measuring storage modulus at a strain of 0.1% and angular frequency of 10 rad/s over a period of 1000 s.

Circular PLGA (85:15, PURAC, Netherlands) films 0.5 mm in thickness and 12 mm in diameter were coated with fibrils by suspending the film overnight in the neutralized collagen solution during fibrillogenesis. The coated films were rinsed three times in Milli-Q and were subsequently air-dried in a fume hood for 1 hour.

The samples were sterilized using ethylene oxide, inserted into 24-well cell culture polystyrene plates (TPP, Switzerland; well diameter 1.5 cm) and filled with 2 ml of McCoy medium, supplemented with 10% fetal bovine serum and gentamicin, with a suspension of human osteoblast cell line SaOs-2. Each well contained 30000 cells (i.e., approximately 17000 cells/cm$^2$). The cells were cultured for 1,3, and 7 days at 37°C in a humidified air atmosphere containing 5% CO2. The cell population densities on days 1 and 3, cell spreading area on day 1, BrdU incorporation on days 1,3 and 7, and viability on day 1 and 7 were evaluated, and were compared with the values of cells on the control microscopic glass coverslips.

On day 1 after seeding, cells were fixed with 70% frozen methanol and stained with a combination of two fluorescence dyes, i.e. Hoechst #33258 (Sigma-Aldrich, Cat. No. B1155; 5µg/ml), which stains the cell nuclei, and Texas Red C2-maleimide (Molecular Probes, Invitrogen, Cat. No. T6008; 20ng/ml), which stains the cell membrane and cytoplasm. The number of cells and their shape on the material surface were evaluated on microphotographs taken under an IX-51 microscope, equipped with a DP 70 digital camera. In addition, the viability was determined by the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen, Molecular Probes, Cat. No. MP03224) according to the manufacturer's protocol. Live and dead cells were then counted on microphotographs taken under the same microscope.

On day 3 after seeding, the presence of β1 integrins (i.e., an important group of cell-matrix adhesion receptors on osteoblasts, supporting their differentiation and their sensitivity to surface properties of the material) and vinculin (protein of focal adhesion plaques, participating in cell–substrate adhesion and stabilizing the focal adhesions) in SaOs-2 cells was evaluated by immunofluorescence staining. The cell were fixed with 70% frozen methanol and were incubated with the following primary antibodies: mouse monoclonal anti-human integrin β1, and mouse monoclonal anti-human vinculin (Chemicon International, Cat. No. MAB1981 and Sigma, Cat. No. V9131, respectively). The secondary antibody was goat anti-mouse F(ab')2 fragment of IgG, conjugated with Alexa Fluor® 488 (Invitrogen, Molecular Probes, Cat. No. A11017). Additionally, the nuclei were stained with Hoechst #33258. After incubation with the secondary antibody and Hoechst, the cells were evaluated under a confocal microscope (Leica SPE, Germany).

On day 7 after seeding, cells were detached using a trypsin-EDTA solution in PBS, and their viability was evaluated using a Vi-CELL XR analyzer (Beckman Coulter, USA).

At each time interval (day 1, 3 and 7 after seeding), the cell proliferation activity was evaluated by incorporating BrdU into the dividing cells (in S-phase) by a Cell Prolifera-
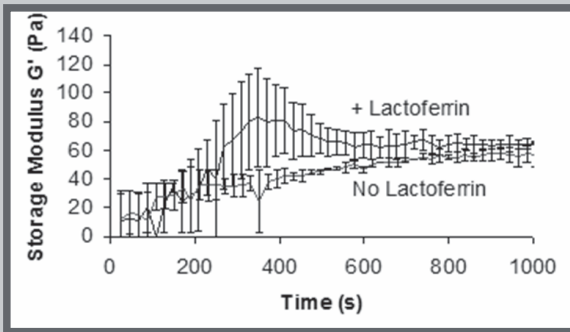
**FIG. 1. Kinetics of collagen gel formation in the presence and absence of lactoferrin. Data presented as mean ± S.D. from 3 measurements.**

tion ELISA kit, BrdU (colorimetric) (Roche, Cat. No. 11 647 229 001). At each time interval, BrdU was added to the culture medium for the last 3 hours of cell cultivation.

The quantitative results were presented as mean ± S.D. (Standard Deviation) or mean ± S.E.M. (Standard Error of Mean). Statistical analyses were performed using SigmaStat (Jandel Corp., U.S.A.). Multiple comparison procedures were made by the One Way Analysis of Variance (ANOVA), Student-Newman-Keuls method. P values equal to or less than 0.05 were considered significant.

## Results and discussions

Rheometric time sweeps (FIG.1) showed that the presence of lactoferrin accelerated collagen gel formation, suggesting an interaction between collagen and lactoferrin. Gels formed in the presence of lactoferrin were not weaker after 1000 s.

On day 1 after seeding, the highest number of cells was found on samples with lactoferrin in comparison with the control microscopic glass coverslips (8380±510 and 5190±610, respectively; P≤0.05; FIG.2). A very similar picture of cell numbers was reached on day 3 after seeding



**FIG. 3. Number of SaOs-2 cells on day 3 after seeding. Data presented as mean ± S.E.M. from 36 measurements. There was no significant difference compared to the control microscopic glass coverslips (P≤0.05)**

(24070±1 610 and 19030±2060 on samples with lactoferrin and glass, respectively), though this difference was not significant (FIG.3). The cells on the tested samples, particularly PLGA with collagen I or with collagen I and lactoferrin, were mostly polygonal and well spread (FIG.4 A-C). The largest cell spreading areas were developed on the samples without
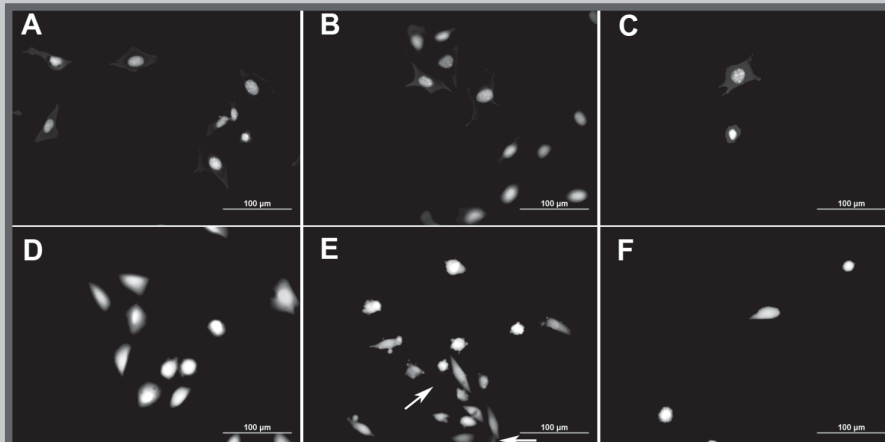


**FIG. 4. Human osteoblast SaOs-2 cells in 1-day-old cultures on samples with lactoferrin (A,D), without lactoferrin (B,E), and on the control microscopic glass coverslips (C,F). The cell nuclei were stained with Hoechst #33258, and the cell membrane and cytoplasm were stained with Texas Red C2-maleimide. The LIVE/DEAD viability/cytotoxicity kit was used (D,E,F) to determine live and dead cells,. Arrows depict dead cells. Olympus IX 51 microscope, objective 20x, DP 70 digital camera, bar = 100 µm.**
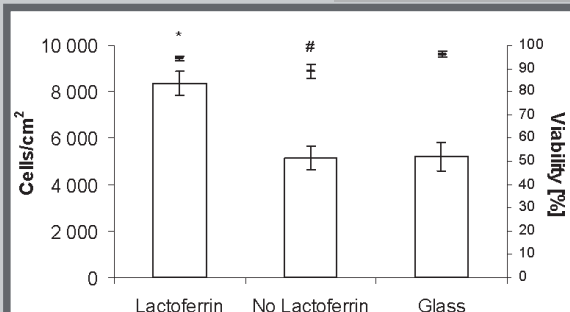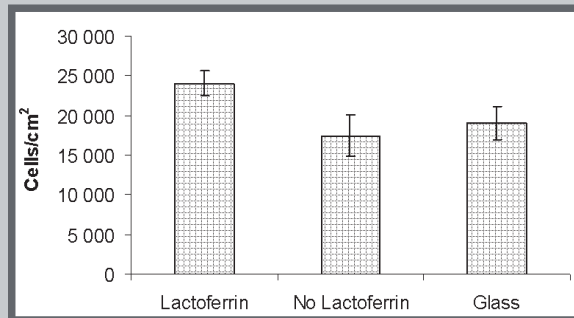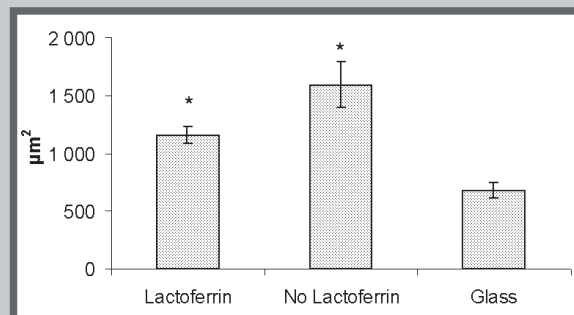


**FIG. 2. Number of SaOs-2 cells and their viability on day 1 after seeding. Data presented as mean ± S.E.M. from 16 measurements. The cell number (*) and viability (#) different significantly from the values on the control microscopic glass coverslips (P≤0.05).**



**FIG. 5. Cell spreading area (µm²) n day 1 after seeding. Data presented as mean ± S.E.M. obtained from 37 to 55 cells. Statistical significance: *P≤0.05 compared to the control microscopic glass coverslips.**
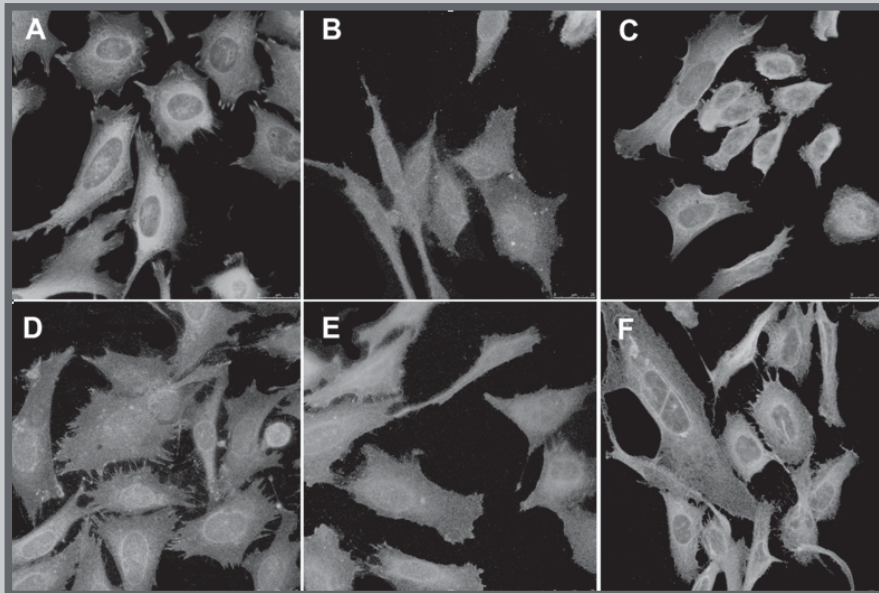
**FIG. 6. Immunofluorescence staining of vinculin (A,B,C) and β1 integrin (D,E,F) in human osteoblast SaOs-2 cells in 3-day-old cultures on samples with lactoferrin (A,D), without lactoferrin (B,E), and on the control microscopic glass coverslips (C,F). The cell nuclei were counterstained with Hoechst #33258. A well-spread cell morphology and a higher number and greater size of focal adhesion complexes on the cell edges were observed on samples with lactoferrin. Confocal laser scanning microscope (Leica SPE, Germany); bar = 25 μm.**

promote various cell activities [6, 7], including mitotic activity [8,9], and to support cell survival [10].

## Conclusion

Lactoferrin accelerated collagen gel formation, increased the cell number on days 1 and 3 post-seeding, maintained the viability of cells, and supported their proliferation.

## Acknowledgments

lactoferrin (i.e., PLGA coated with collagen I only) on day 1 after seeding (1590±200 μm$^2$). However, cells on samples with lactoferrin have bigger spreading areas than the cells on the control microscopic glass coverslips (1160±70 and 680±70 μm$^2$, respectively; FIG.5). In addition, the cells on the lactoferrin-containing samples had more numerous and better developed focal adhesion plaques, as revealed by immunofluorescence staining of vinculin and β1-integrins (FIG.6).

On days 1, 3 and 7, BrdU incorporation into the proliferating cells was assessed (BrdU incorporation into the newly synthesized DNA was measured during the last 3 h of cell cultivation). In each time interval, the cells on the lactoferrin samples showed better proliferation than the cells the control microscopic glass coverslips (FIG.7). The cell viability was high, ranging between 89 and 97% on day 1 (FIG.2, 4 D-F), and between 93 and 96% on day 7 after seeding. All these findings are in good correlation with the results in the literature, where lactoferrin has been found to

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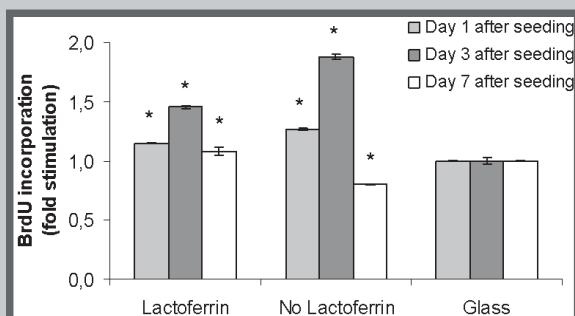
**FIG. 7. Effects of lactoferrin on BrdU incorporation into proliferating cells. Data presented as mean ± S.E.M. from 4 measurements. Statistical significance: *P≤ 0.05 compared to the values on the control microscopic glass coverslips.**