**In Vitro Biocompatibility of Piezoelectric $K_{0.5}Na_{0.5}NbO_3$ Thin Films on Platinized Silicon Substrates**

Nikolai Helth Gauka, Quy-Susan Huynh, Anishchal A. Pratap, Mari-Ann Einarsrud, Tor Grande, R. M. Damian Holsinger,* and Julia Glaum*

**ABSTRACT:** Lead-free piezoelectric ceramics like $K_{0.5}Na_{0.5}NbO_3$ (KNN) represent an emerging class of biomaterials for medical technology, as they can be used as components in implantable microelectromechanical systems (MEMS) and bioactive scaffolds for tissue stimulation. Such functional materials can act as working components in future in vivo devices, and their addition to current implant designs can greatly improve the biological interaction between host and implant. Despite this, only a few reports have studied the biocompatibility of these materials with living cells. In this work, we investigate the biological response of two different cell lines grown on KNN thin films, and demonstrate excellent biocompatibility of the KNN films with the cells. Undoped and 0.5 mol % CaTiO$_3$-doped KNN thin films with nanometer-sized roughness were deposited on platinized silicon (SiPt) substrates, and cell proliferation, viability, and morphology of human 161BR fibroblast cells and rat Schwann cells grown on the KNN films and SiPt substrates were investigated and compared to glass control samples. The results show that proliferation rates for the cells grown on the KNN thin films were equally high or higher than those on the glass control samples, and no cytotoxic effect from either the films or the substrate was observed. The work demonstrates that KNN thin films on SiPt substrates are very promising candidates for components in implantable medical devices.

**KEYWORDS:** KNN thin films, platinized silicon, topography, wetting, microstructure, cell viability

**INTRODUCTION**

Piezoelectric ceramics are utilized in a wide range of electromechanical technologies due to their ability to passively transduce electrical and mechanical signals. This characteristic makes them suitable for a wide range of applications, e.g., as transducers for ultrasound imaging, for structural health monitoring of airplanes, or simply as a speaker in a mobile phone. In contrast to electromagnetic systems, piezoelectric devices can transduce electrical and mechanical signals. This characteristic makes them versatile and allows them to be used in a wide range of applications.

Piezo- and ferroelectric materials also hold great potential for biomedical applications as they can be utilized as active scaffolds for tissue engineering and energy harvesters, sensors and actuators for implantable microelectronic systems as well as for biosensing and -patterning.

**ABSTRACT:** Lead-free piezoelectric ceramics like $K_{0.5}Na_{0.5}NbO_3$ (KNN) represent an emerging class of biomaterials for medical technology, as they can be used as components in implantable microelectromechanical systems (MEMS) and bioactive scaffolds for tissue stimulation. Such functional materials can act as working components in future in vivo devices, and their addition to current implant designs can greatly improve the biological interaction between host and implant. Despite this, only a few reports have studied the biocompatibility of these materials with living cells. In this work, we investigate the biological response of two different cell lines grown on KNN thin films, and demonstrate excellent biocompatibility of the KNN films with the cells. Undoped and 0.5 mol % CaTiO$_3$-doped KNN thin films with nanometer-sized roughness were deposited on platinized silicon (SiPt) substrates, and cell proliferation, viability, and morphology of human 161BR fibroblast cells and rat Schwann cells grown on the KNN films and SiPt substrates were investigated and compared to glass control samples. The results show that proliferation rates for the cells grown on the KNN thin films were equally high or higher than those on the glass control samples, and no cytotoxic effect from either the films or the substrate was observed. The work demonstrates that KNN thin films on SiPt substrates are very promising candidates for components in implantable medical devices.

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hydroxyapatite. One study has also conducted in vivo histological assays of KNN samples in rat thighs and found no sign of inflammation after 7 days of implantation.

Here, we report on in vitro biocompatibility of KNN thin films on platinitized silicon (SiPt) substrates. Undoped and 0.5 mol % CaTiO₃-doped KNN films were synthesized by aqueous chemical solution deposition (CSD) on SiPt substrates based on a previously reported processing route. The biocompatibility of these films was assessed through proliferation, morphology, and viability assays using human 161BR fibroblast cells and rat (glial) Schwann cells. To deconvolute the biological effects from the KNN films and the substrate, SiPt substrates with no deposited film were also tested. Glass coverslips were used as negative controls as they have been reported to support cell attachment and proliferation for both fibroblast and glial cells. The results obtained demonstrate that KNN thin films and SiPt substrates exhibit excellent biocompatibility with both cell lines, and we discuss these findings in the light of bulk and surface properties of the materials.

### MATERIALS AND METHODS

#### Thin Film Synthesis.

Undoped KNN (K₀.₅Na₀.₅NbO₃) and 0.5 mol % CaTiO₃ doped KNN films (K₀.₄⁹₇₅Na₀.₄⁹₇₅Ca₀.₀₅Nb₀.⁹⁹₅Ti₀.₀₅O₃) were prepared using aqueous precursor solutions described elsewhere. The solutions were prepared by precipitating niobic acid from an aqueous solution of NH₄NO₃(C₆H₇O₄)₂·4H₂O (99.99%, Sigma-Aldrich, St. Louis, MO, U.S.A.) using ammonia solution (25 wt %, VWR Chemicals, Radnor, PA, U.S.A.). The niobic acid precipitate was dissolved in deionized water with DL-malic acid (99%, Sigma-Aldrich) in a molar ratio of 1:3. The pH of the Ca²⁺ and Ti⁴⁺ solutions were adjusted to 8 by addition of ammonia solution (25 wt %, VWR Chemicals, Radnor, PA, U.S.A.). Film thickness was determined by scanning electron microscopy (SEM, Ultra 55, Carl Zeiss AG, Oberkochen, Germany) operating at 10 kV acceleration voltage and using an in-lens detector. The surface roughness of the films and the SiPt substrates was studied using atomic force microscopy (AFM, Multimode V, Veeco Metrology, Plainview, NY, U.S.A.).

The hydrophobicity (wettability) of the films and substrate was analyzed by contact angle measurements of deionized water droplets at 37 °C (DSEA1000, KRÜSS, Hamburg, Germany).

#### Cell Culture.

Human 161BR fibroblast cells were cultured in 3 mL of media (Eagle’s MEM with 15% fetal bovine serum (FBS), 2 mM L-glutamine, 1% nonessential amino acids (NEAA), 1% Penicillin-Streptomycin (Pen-Strep)) at 37 °C with 5% CO₂ in a T25 flask until confluent. Rat Schwann cells were cultured in 3 mL of media (Ham’s F12 with 10% FBS, 2 mM L-glutamine, and 1% Pen-Strep) at 37 °C with 5% CO₂ in a T25 flask until confluent. All media and components were purchased from Sigma-Aldrich (Seven Hills, NSW, Australia). The media for both cell lines were changed every 3 days. When confluent, the cells were lifted from the T25 flask using 0.025% trypsin (Gibco, Cat no. 15400054 in 1x phosphate buffered saline (PBS) no calcium, no magnesium) and seeded onto glass coverslips (control, Hurst Scientific, WA, Australia), SiPt, KNN/SiPt and KNN-CaTiO₃/SiPt in a 24-well plate along with 500 μL of media. Three samples were used for each material and control.

#### Cell Proliferation Assay (MTT).

Cell proliferation of 161BR fibroblast and Schwann cells on SiPt substrates and KNN thin films was screened using an MTT colorimetric assay. The cell densities seeded for the proliferation assay were 2.0 × 10⁴ cells per well (161BR fibroblast cells) and 4.3 × 10⁴ cells per well (Schwann cells). The cells were grown in media at 37 °C with 5% CO₂ for 1, 3, 5, and 7 days (triplicates at each point). The media was changed every 2 days after seeding. The colorimetric assay was performed after 1, 3, 5, and 7 days. The culture media was removed, and 50 μL of an MTT solution (5 mg mL⁻¹ MTT, VWR/Amersco, Cat no. 97062-380 in 1x PBS (1%)) and 450 μL of serum-free Eagle’s MEM (161BR fibroblast cells) or serum-free Ham’s F12 medium (Schwann cells) were added to the wells. The 24-well plate was incubated for 4 h at 37 °C with 5% CO₂. Following this, the MTT solution was carefully removed and the formed formazan crystals were dissolved in an MTT solvent (4 mM HCl and 0.1% Nonidet P-40 (VWR/Amersco) in isopropanol). The plate was wrapped in foil and left on a rocker for 10 min. The MTT solvent was then collected and placed into a 96-well plate for spectrophotometric analysis, measuring absorption at 560 nm wavelength (Hitex Chameleon Multilabel plate reader, Turku, Finland). Statistical analysis of the data was performed using one-way ANOVA and statistical significance was set at p < 0.05.

#### Cell Morphology Assay.

Cell morphology of 161BR fibroblast cells and Schwann cells on SiPt substrates and KNN thin films was studied using SEM. The cell densities seeded for SEM imaging were 3.9 × 10³ cells per well (161BR fibroblast cells) and 6.3 × 10³ cells per well (Schwann cells). The cells were grown in media at 37 °C with 5% CO₂ for 5 days (161BR fibroblast cells) and 3 days (Schwann cells). For the 161BR fibroblast cells, the media was changed 2 days after seeding. After 3–5 days, the cells were washed twice with 500 μL of warm 1x PBS and fixed using 300 μL glutaraldehyde (3% in 1x PBS, preheated to 37 °C, Sigma-Aldrich). The cells were stored in the plate with glutaraldehyde for 20 h at 4 °C in a zip lock bag to prevent loss of glutaraldehyde through evaporation. The following day, the glutaraldehyde was removed, and the cells were washed twice with 300 μL of 1x PBS at 4 °C for 10 min. Prior to dehydration, the cells were hydrated twice with deionized water for 10 min. The cells were then gradually dehydrated using single washes of 30, 50, 70, 80, and 90% (v/v) ethanol and two washes with 100% ethanol, with each wash lasting 12 min. Following this, the dehydrated cells were left for 24 h for the ethanol to completely evaporate. The samples were imaged using SEM (TM3030, Hitachi, Tokyo, Japan).

#### Cell Viability Assay (Immunofluorescence).

Cell viability of 161BR fibroblast and Schwann cells on SiPt substrates and KNN thin films was studied using immunofluorescent microscopy. The cell densities seeded for immunofluorescent microscopy were 1.3 × 10⁴ cells per well (161BR fibroblast cells) and 6.3 × 10⁴ cells per well (Schwann cells). The cells were grown in media at 37 °C with 5% CO₂ for 5 days (161BR fibroblast cells) and 3 days (Schwann cells). For the 161BR fibroblast cells, the media was changed 2 days after seeding. When cells reached ~90% confluence (after 3–5 days), the
cells were washed 2 times with 600 μL of 1× PBS and fixed using 300 μL of paraformaldehyde (4% (w/v), pH 7.4) warmed to 37 °C. The cells were then permeabilized with 300 μL of Triton X-100 in 1× PBS (0.1%) for 15 min at room temperature. Permeabilization facilitates antibody penetration into the cells. Following this, the cells were washed three times with 300 μL of 1× PBS. The cells were blocked using 300 μL of bovine serum albumin (BSA) in 1× PBS (1%) for 60 min at room temperature to prevent nonspecific antibody–protein interaction. Cells were immunostained with 300 μL of monoclonal β-tubulin primary antibody (1:5000 dilution in PBS, Sigma-Aldrich, Cat no. T7816) which was left in for 16 h at 4 °C. The next day, the primary antibody was removed, and the cells were washed three times with 300 μL of 1× PBS. The β-tubulin primary antibodies were detected by adding 300 μL of Alexa Fluor 488-conjugated rabbit antimouse IgG (H+L) secondary antibody (1:5000 dilution in PBS, Life Technologies, Cat no. A1005534) for 45 min in room temperature and away from light. Following this, the cells were washed once with 300 μL of 1× PBS. In order to stain cell nuclei, 50 μL of 4′,6-diamidino-2-phenylindole (DAPI, 0.1 g L⁻¹ PBS, Sigma-Aldrich, Cat no. D9542-10MG) was added for 3 min at room temperature. The cells were then washed twice with 300 μL of 1× PBS, and glass coverslips were mounted on the samples using dibutyl phthalate in xylene (DPX). The immunostained cells were imaged using confocal microscopy (LSM 800, Carl Zeiss AG).

RESULTS

Microstructure and Phase Composition of the KNN Films and SiPt Substrates. Cross sectional SEM micrographs of the KNN and KNN-CaTiO₃ thin films prepared from CSD on SiPt demonstrate that the KNN films were dense, homogeneous, and without cracks, pinholes, or other macroscopic defects (Figure 1). The thickness of the KNN films fabricated from 6 depositions was ~50 nm for both compositions. GIXRD patterns of the KNN and KNN-CaTiO₃ thin films are presented in Figure S1 in the Supporting Information. All of the Bragg reflections can be assigned to monoclinic K₀.₅Na₀.₅NbO₃ 3¹ and the substrate, confirming that single phase KNN was deposited on SiPt substrates using the aqueous CSD route.25,26

Figure 2. AFM topography images of (a) glass coverslips, (b) SiPt substrates, (c) KNN thin films, and (d) KNN-CaTiO₃ thin films. The ranges of the scalebars in panels a–d differ. (e) The RMS roughness of the samples calculated from AFM measurements.

Figure 3. Contact angles of distilled water drops on top of the samples at 37 °C. The values are averages from measurements on 10 drops.

The contact angles of water droplets at 37 °C on SiPt substrates, KNN thin films and KNN-CaTiO₃ thin films as well as glass coverslips are presented in Figure 3. All samples show hydrophilic surfaces without significant difference between the materials.
**Biocompatibility.** Cell proliferation rates after 1, 3, 5, and 7 days for 161BR fibroblast and Schwann cells grown on SiPt substrates, KNN thin films, KNN-CaTiO3 thin films, and glass coverslips (control) are shown in Figure 4a,b, respectively.

Both cell lines adhered well to all of the materials tested. The 161BR fibroblast cells were observed to spread out more on the SiPt substrates and the KNN thin films compared to the cells grown on the control and the KNN-CaTiO3 thin films. A higher density of Schwann cells was observed growing on the SiPt substrates and the KNN thin films compared to the growth of cells on the control and the KNN-CaTiO3 thin films.

![Figure 4. Proliferation rates of (a) human 161BR fibroblast and (b) rat Schwann cells after being grown for 1, 3, 5, and 7 days on glass coverslips (control), SiPt substrates, KNN thin films and KNN-CaTiO3 thin films. Statistical significance is indicated (* = p < 0.05, ** = p < 0.01). ## refers to a difference of p < 0.01 between SiPt and each of the other samples.](image)

### DISCUSSION

The variations in viability and proliferation rate of cells grown on different materials can be attributed to surface properties (e.g., topography, hydrophobicity, and surface charges) and/or to bulk properties (e.g., ion release and stiffness) of the substrate materials. In this study we assessed the biocompatibility properties of various sample types in vitro to determine their suitability for incorporation into biomedical devices.

**Surface Properties.** Surface topography can facilitate cell orientation/adhesion, morphology, differentiation and proliferation.\(^{32–34}\) Manipulation of cell alignment and growth using micrometer-sized topography has been demonstrated for fibroblast\(^{35}\) and glial cells.\(^{36}\) Nanometer-sized topography, however, indirectly affects cell behavior by influencing protein adsorption and by resembling the natural extracellular matrix.\(^{37}\) Different proteins have differing affinities for size and shape of surface topographic features, and the reported effects vary. Nanometer-sized topography has been demonstrated to influence fibroblast cells with cell proliferation and spreading increasing with decreasing surface roughness in the ranges of 23–0.7 nm\(^ {38}\) and 95–13 nm.\(^ {39}\) The research on glial cells is more nuanced, and these cells have been found to be unaffected by nanotopography in the range of 0.7–23 nm.\(^ {38}\) Instead, they seem to prefer “grassy” regions with structures of height 230 nm and diameter of 60 nm over smoother regions with imprints of 115 nm depth and 100–250 nm width.\(^ {30}\) In the present study, the average surface roughness (S\(_{a}\)) of the KNN (3.3 ± 0.5 nm) and KNN-CaTiO3 films (2.9 ± 0.5 nm) was found to be greater than the roughness of the SiPt substrates (0.7 ± 0.1 nm) and glass coverslips (0.3 ± 0.2 nm). This difference between the coated and uncoated substrates is related to the growth of grains in the ceramic films during heat treatment. The slightly larger roughness of the KNN compared to the KNN-CaTiO3 films is caused by a larger average grain size in undoped KNN as reported previously.\(^ {20}\) The roughness of the samples in the present study does not appear to influence cell behavior, as cells proliferated and spread equally well on the glass controls, SiPt substrates and the KNN films (Figures 4 and 5). Compared to other studies, these results demonstrate that the surface topography differences in our work (0.3–3.3 nm) are too small to influence the behavior of fibroblast and glial cells. Glial cells have been reported to be unaffected by nanosized differences in the range of 0.7–23 nm,\(^ {38}\) and our results suggest that there is also a lower limit for fibroblast cells, where differences in the surface roughness do not influence cell behavior.

Surface hydrophobicity of biomaterials is directly linked to biocompatibility since the proteins forming binding sites for cells (e.g., fibrin and albumin) adhere to the biomaterial using hydrophobic domains.\(^ {32,41}\) However, a surface that is too hydrophobic will cause protein relaxation and loss of biological activity. The composition and orientation of proteins binding to the surface is governed by the surface hydrophobicity, and different cell lines have different preferences regarding protein binding sites. Fibroblast cells have been reported to have their

![Figure 5. SEM micrographs and confocal immunofluorescence images of 161BR fibroblast cells (top two rows) and Schwann cells (bottom two rows) after being grown on glass coverslips, SiPt substrates, KNN thin films and KNN-CaTiO3 thin films for 5 (161BR fibroblast cells) and 3 days (Schwann cells). Cell cytoskeletons and nuclei are stained with β-tubulin (green) and DAPI (blue), respectively.](image)
peak proliferation rate on surfaces with contact angles (θc) around 60°−70°.21,42−44 The case is not so clear for glial cells, and increased proliferation has been observed for both hydrophobic over hydrophilic surfaces45 as well as hydrophilic over hydrophobic surfaces.46,47 The surface hydrophobicity measurement in this work demonstrates that all of the samples are moderately hydrophilic with contact angles between 50° and 60° (Figure 4). These values are in agreement with literature on other ceramics and metals.48 The observed differences in surface hydrophobicity between the samples in this study are minor and do not seem to have a significant impact on cell behavior. However, overall, cell adhesion, viability and proliferation of both cell lines were good, suggesting that a surface hydrophobicity of 50°−60° is suitable for growth of both fibroblast and glial cells. This is in agreement with previous studies on fibroblast cells42−44 and suggests that glial cells are compatible with moderately hydrophilic surfaces in addition to more extreme wetting angles.45−47 The wetting behavior in an in vitro scenario, however, might vary, as this characteristic is quite sensitive to many factors such as changes in topography or physicochemical conditions.49

Electric surface potentials on poled KNN ceramics have been shown to promote higher cell proliferation rates compared to unpoled KNN ceramics.16,18−20 The strength of this surface polarization has been reported to correlate positively with cell proliferation,20 but the optimal direction of polarization for cell growth seems to vary between cell lines.18,19 A macroscopic polarization of ferroelectric samples can be obtained by electrical poling and KNN has been reported to retain most of the ferroelectric functionality after γ-ray sterilization up to 3000 kGy.30 The KNN films in this study were not electrically poled before cell testing and should therefore exhibit a random ferroelectric domain distribution. Even though the material is in its polar state and a polarization state can be assigned to each domain, the sum of all domain contributions to the macroscopic polarization cancels out due to the random orientation of the domains. The ferroelectric domains of the unpoled films are limited to the sizes of the grains (<100 nm) and are thus expected to be too small to influence cell behavior. The effect of both poling and mechanical stimulation of the KNN samples is an interesting approach that should be considered in future studies evaluating the biocompatibility of these materials.

Bulk properties. The leaching of ions, molecules or particles from a material can be harmful to cells if the material releases products that are toxic. Release processes from a bulk material can be slow, and observing the chronic cytotoxic effect of materials in short-term studies is usually impossible.51,52 This has been exemplified with Pb(Zr,Ti)O3-based ceramics that seemingly show no cytotoxicity in vitro and in vivo in short-term experiments.53 In contrast to that, leaching studies performed on powders show higher release rates due to their higher surface area, providing a better indicator for long-term toxicity effects. In the present study, several different materials have been brought in contact with cells. The SiPt substrates, mostly comprised of Si and platinum metal, are expected to be chemically inert in the cell culture medium and to not release ions during in vitro studies. For KNN, ion release assays have been performed on powders in several studies.31−23,55 The release of K+ and Na+ is 1−2 orders of magnitude higher than that of Nb55 due to the lower ionic strength of these ions. For 0.5 wt % powder suspensions in distilled water at room temperature the K+/Na+ release is 1−3 mmol L−1.55 These values are not negligible, and studies have linked the release of ions from KNN powders to a reduction in the rate of proliferation.31,32 Although all cations in KNN (including Ca2+ and Ti4+ in the doped KNN composition in this work) are nontoxic in small concentrations,10,54,55 local concentrations of leached ions can be high. Leaching of Na+ and K+ from soda-lime glass in water has been reported.56 Assuming the release of alkali metals is the cause of differences observed in the cell experiments, the SiPt samples should show the best performance, which is indeed the case for most of the time points investigated in both cell line experiments (Figure 4), whereas the alkali-containing samples perform mostly on par with one another.

Material stiffness has been demonstrated to influence cell adhesion, spreading, proliferation, and differentiation.57,58 Most reports have studied the effect of substrate stiffness in the range of 103−106 Pa. In general, increased substrate stiffness is associated with enhanced proliferation, enlarged cell spreading and higher proliferation rates. Glial cells fail to grow on substrates with stiffness in the range 102−103 Pa but overrun the glass control samples (>10 GPa) in the same experiment.59 However, not all cell types show a monotonic dependence on substrate stiffness. Fibroblast cells exhibit increased proliferation and spreading with increasing substrate stiffness in the range from 100 Pa to 100 kPa, but they also start to produce stress fibers above 10 kPa.60 Yet, fibroblast cells have been reported to proliferate well on glass substrates.61−28 The bulk Young’s moduli of the materials used in the current study are reported to be in the range of 80−170 GPa,61−63 while for KNN films on SiPt, values between 70 and 100 GPa can be found.64 These values are much higher than the values typically used when studying the effect of substrate stiffness on cells and it is not clear from the literature whether the differences in such a high stiffness regime will influence cell behavior. Moreover, the three Si-based samples in this study (SiPt, KNN/SiPt, and KNN-CaTiO3/SiPt) and especially the two KNN films should have almost identical stiffnesses, as the mechanical characteristics of the film is expected to be dominated by the properties of the substrate. This suggests that the differences observed between these samples in the in vitro assays are not caused by the materials’ stiffness.

CONCLUSION

The in vitro biocompatibility of KNN films was investigated using human 161BR fibroblast and rat Schwann cells. Undoped and 0.5 mol % CaTiO3-doped KNN thin films were deposited on SiPt substrates by aqueous CSD and the proliferation, morphology and viability of cells grown on the films were assessed. Both KNN film compositions support cell attachment, spreading and proliferation equally well or better than the glass control samples. The results further suggest that fibroblast and glial cells are indifferent to differences in surface roughness between 0.3 and 3.3 nm and that these cell types respond well to moderately hydrophilic surfaces (θc ≈ 50−60°). When evaluated together with previous work on biocompatibility of KNN ceramics, our findings strongly support the description of KNN as a nontoxic ceramic that exhibits biocompatibility with a wide range of cell lines. To the best of our knowledge, our results are the first to establish platinized silicon substrates as nontoxic. This work demonstrates that KNN thin films and SiPt substrates exhibit excellent in vitro biocompatibility and that KNN films...
hold the potential as functional components in implantable medical devices.

**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsabm.0c01111. XRD patterns of KNN films (PDF)

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**Author Contributions**

N.H.G., Q.S.H., R.M.D.H., and J.G. conceived and designed the experiments. N.H.G. synthesized the thin films and performed SEM and XRD on the thin films. N.H.G. and Q.S.H. cultured cells, performed the proliferation assays, and imaged the cells with SEM. N.H.G., Q.S.H., and A.A.P. stained the cells. A.A.P. imaged the cells with confocal microscope. All authors analyzed the data. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

**Notes**

The authors declare no competing financial interest.

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