

An Efficient Covalent Coating on Glass Slides for Preparation of Optical Oligonucleotide Microarrays

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ABSTRACT

Objective(s): Microarrays are potential analyzing tools for genomics and proteomics researches, which is in need of suitable substrate for coating and also hybridization of biomolecules.

Materials and Methods: In this research, a thin film of oxidized agarose was prepared on the glass slides which previously coated with poly-L-lysine (PLL). Some of the aldehyde groups of the activated agarose linked covalently to PLL amine groups; also bound to the amino groups of biomolecules. These linkages were fixed by UV irradiation. The prepared substrates were compared to only agarose-coated and PLL-coated slides.

Results: Results on atomic force microscope (AFM) demonstrated that agarose provided three-dimensional surface which had higher loading and binding capacity for biomolecules than PLL-coated surface which had two-dimensional surface. In addition, the signal-to-noise ratio in hybridization reactions performed on the agarose-PLL coated substrates increased two fold and four fold compared to agarose and PLL coated substrates, respectively.

Conclusion: The agarose-PLL microarrays had the highest signal (2546) and lowest background signal (205) in hybridization, suggesting that the prepared slides are suitable in analyzing wide concentration range of analytes.

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Introduction

Microarray technology is a potential tool in biomolecular researches, which provide high sensitivity and specificity in genomic and proteomic tests (1-4). One of the important challenges in this technology is the surface chemistry of substrate to be compatible with immobilization of probe biomolecules (5). Glass, most widely used substrate of these biosensors, is inexpensive and inert and has low background signal under fluorescence detection (6, 7). Moreover, glass can be easily modified (8, 9) to increase binding capacity, hybridization efficiency, spot uniformity and stability of immobilized probes (5). Different modification methods activate glass surface for attachment of biomolecules as diagnostic probes. Depending on the properties of the coating, physical or chemical interactions may occur between probe and modified surface of the glass. Poly-L-lysine (PLL) and aminosilane are physically attached to glass substrate and do not require probe modification (9, 10). However, these substrates have low binding capacity and flat surfaces which result in steric limitations in hybridization of target to probes (10). To solve this issue, probes can be

elevated from flat surface. This actually may increase the binding capacity of the glass (11, 12). Polymers such as poly acrylamide or agarose can act as dendrimetric spacers which can elevate probes from substrate and show high hybridization efficiency (13-17).

Agarose is inexpensive, non-toxic (18, 19) and commercially available material. Agarose-coated glass slides can provide aldehyde groups which bind to amino-modified DNA probes; also DNA probes can attach to agarose-coated glass surface using ultraviolet (UV) radiation (19).

In this research, we therefore prepared PLL, agarose and PLL/agarose coatings on the standard glass slides and immobilized using UV radiation. The prepared slides were compared together and finally the obtained results as a potential substrate for DNA microarray.

Experimental procedure

Materials

Microscope glass slides (25.4 mm × 76.2 mm × 1 mm) and glass cover slips were purchased from Sigma-Aldrich. PLL (poly-L-lysine mol wt 30000-70000) was purchased from Sigma and agarose was

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Table 1. DNA probes that was spotted on the fabricated substrates

Probe name	Probe sequence
P1	5'-CCGCTATGACATCGACATGACCAAGTGCATCTACTGC-3'
P2	5'-GGAAATATGGAAGCACTTTGACGACAGACGAAATGGGTAT-3'
P7	5'-AGACAGATGAGGAGTTTGATGCTCGCTGGGTAACATACTT-3'
P8	5'-AACAAAGAGAATAGTAGGCTGCATCTGTGAAGAGGACAATACCA-3'
P26	5'-GCTGTACCGAACGGATCTTGCTCTTATTCTCTGTGCTG-3'

provided by Roche. Betaine, sodium diodecyl sulfate (SDS), succinic anhydride (SA), N-methyl 2-pyrrolidone (NMP), boric acid, sodium hydroxide, ethanol, bovine serum albumin (BSA), formamide and salmon sperm DNA were purchased from Sigma. Saline sodium citrate was provided from Ambion. Sodium periodate was purchased from Fluka. DNA extraction kits were purchased from Tadbir Fan Azma Co. Cy5mono-reactive dye was purchased from GE-Healthcare. Finally, DNA labeling kits were provided by Agilent Technologies Co.

Preparation of polymer film-coated slides

Three different polymer layers were coated on the standard glass slides: (i) poly-L-lysine (PLL) coating, (ii) Agarose coating, and (iii) Agarose layer on the PLL coating.

PLL was coated on the glass microscope slides according to the standard protocol. Agarose layer was also coated on the both unmodified glass slides and PLL-coated glass slides according to method described by Dufva *et al* (19).

Preparation of microarrays

DNA probes, which have 35-40 bp long (Table 1) was spotted on the fabricated substrates using Q-array mini system (Genetix, Germany). Printing solution, containing 150 mM of phosphate buffer, pH 8.5 and 3X saline sodium citrate with 1.5 M betaine was prepared. Dried probes were suspended in the mixture of equal volume of printing buffer and dH₂O to obtain final concentration ~ 200 ng/μl.

Substrates were printed under 40-50% relative humidity, and then dried in air for 10 min. The probes were spotted in quadruplicate on the substrates. The DNA probes were immobilized on the slides by UV radiation at 254 nm using a CL-1000 UV crosslinker (UVP Inc.) for 3 min. Then, arrays were immersed in 1% sodium diodecylsulphate (SDS) for 30 sec. Finally, the slides were dried using compressed nitrogen gas.

Morphological characterization of the coated slides

The micro-scale micrographs of the spotted and unspotted glass slides were obtained using AIS2100 Scanning Electron Microscopy (Seron Technology Inc., Korea). A Hysitron Inc. (USA) Triboscope® Nanomechanical test instrument with 2D transducer, complete software and Berkovich diamond indenter was used to measure reduced elastic modulus (E_r) and hardness (H) of the coated and uncoated glass slides. Five measurements were performed in the peripheral and central parts of the slides. The AFM (Atomic Force Microscope) part, a Nanoscope E (Digital Instruments, USA) was used to obtain images of the surface. The AFM images were analyzed using Nanoscope® software version III 5.12r2. Mean roughness (R_a), root-mean square roughness (R_{ms}), maximum height (R_{max}) and 3D surface area (R_{area}) were calculated using this software.

Target preparation and hybridization

Target preparation and labeling

Genomic DNA was extracted from peripheral blood using TadbirFan Azma DNA extraction kit (Iran). For labeling genomic DNA, 2 μg of DNA was added to dH₂O to bring total volume to 21 μl. Then, 20 μl of 2.5X random primer/ reaction buffer was added. The mixture was boiled for 5 min, and then placed on ice. 5 μl of 10X dNTP was added on ice. Then, 3 μl of cy5-dCTP dye and 1 μl of Klenow fragment were added. The mixture was incubated at 37°C for 2 hr. The reaction was stopped by adding 5 μl of 0.5 M EDTA at pH 8.0.

Hybridization

Hybridization solution was prepared according to Table 2.

Pre-hybridization of the slides was done to eliminate non-specific binding of target to the slides. The slides were immersed in hybridization solution and incubated at 42°C for 1 hr in a water bath. Then, the pre-hybridized slides were washed in dH₂O and dried using compressed nitrogen gas.

Table 2. Recipe for preparing 100 ml of 1X hybridization solution

Ingredient	Amount required	Final concentration
20X SSC	25 ml	5X SSC
SDS	0.1 g	0.1% (w/v)
BSA	1.0 g	1.0% (w/v)
Formamide	50 ml	50% (v/v)
Salmon sperm DNA	10 mg	0.01% (w/v)
Deionized water	Fill to 100 ml	

SDS: sodium diodecyl sulfate

Table 3. Surface analysis data of the samples

Sample	R _a (nm)	R _{ms} (nm)	R _{max} (nm)	R _{area} (μm) ²
Agarose on PLLcoated slide	7.086	9.025	62.433	25.059
Agarose on glass slide	7.334	9.156	63.601	25.063
PLL coated slide	0.869	1.572	20.488	25.005

PPL: poly-L-lysine

Labeled DNA target was diluted in hybridization solution to 10 nM final concentrations. The microarrays were hybridized with appropriate amount of target under cover slips in incubator at 42°C for overnight. Then, the slides were immersed in the solution 2X SSC and 0.1% SDS and washed in 1X SSC for 1 min, in 0.2X SSC for 1 min and 0.05X SSC for 1-2 sec and finally rinsed in dH₂O and dried using compressed nitrogen gas.

Microarray quantification

The hybridized microarrays were scanned using Scan Array GX Microarray Scanner (Perkin Elmer). Spots were quantified from the images generated from Scan Array GX Microarray software (Perkin Elmer) using Scan Analyze software version 2.5. For calculating signal-to-noise ratio (SNR) of spots, the following formula was employed:

$$SNR = \frac{(SM - BM)}{\text{standard deviation of background}}$$

Where SM was mean signal of four spots replicated of each probe when hybridized to cy5 labeled target and BM was mean background signal.

Results

The principle of different coatings linked with biomolecules

Poly-L-lysine (PLL) used to coat glass slides for preparing microarrays can attach to glass slides stronger than agarose layer. However, PLL-coated surfaces have low binding capacity for biomolecules. Activated agarose provided a thin layer on the glass with active aldehyde groups which can bind to various biomolecules, such as DNA, proteins, peptides and phospholipids. In addition of providing active groups on the surface, agarose could perform a physical function. It was shown that agarose provides a uniform three-dimensional solid support for dynamic attaching of biomolecules. Aldehyde groups in activated agarose chemically bound to amino groups in PLL structure. Therefore, agarose could attach to modified slides stronger than unmodified slides.

Consequently, higher stability in hybridization reactions and higher signal-to-noise ratio was provided.

Surface analysis of polymer film-coated slides

The surface profiles of the coated and uncoated glasses were characterized by AFM showed that agarose provided three-dimensional thin film on the

unmodified and PLL-coated glass slides (Figures 1, 2). The mean roughness (R_a), root-mean square roughness (R_{ms}), maximum height (R_{max}) and 3D surface area (R_{area}) for agarose-PLL coated, agarose coated and PLL-coated glass were reported in Table 3. The section analysis of agarose-PLL coated, agarose coated and PLL-coated slides revealed that the vertical distance were 35.520 and 33.504 nm respectively, while vertical distance of PLL-coated surface was 0.817 nm.

The mean reduced elastic modulus (E_r) and mean hardness (H) were calculated from five indentation performed in peripheral and central parts of the surface. These data were reported in the Table 4 for agarose on PLL-coated slides, agarose on unmodified glass and unmodified glass. Although, agarose coatings were applied manually to the surface, five different measurements showed agarose layers were relatively uniform.

Spot uniformity and morphological analysis of prepared microarrays

SEM analysis of the agarose on the glass slides showed a porous film (Figure 4). This micrograph confirmed its orientation, parallel to the glass surface.

The SEM micrograph of the prepared microarrays on three different coated substrates showed spots written on the agarose layers were more regular than spots on the PLL-coated slides in shape and size [Figure 5(a), 5(b) and 5(c)]. Some spots on the PLL-coated substrate had an inappropriate quality and were ununiformed in diameter. It is worth mentioning that there was best quality of spots on the agarose. Moreover, the spot diameter was between 200-250 μm.

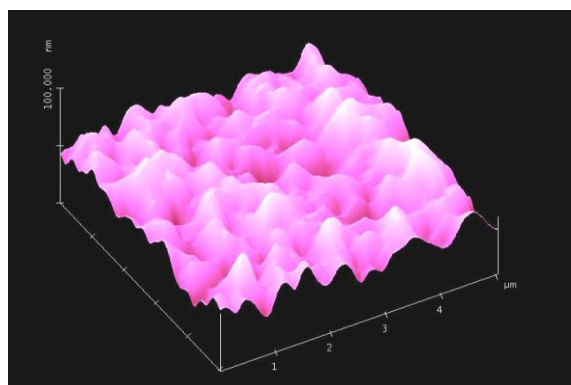
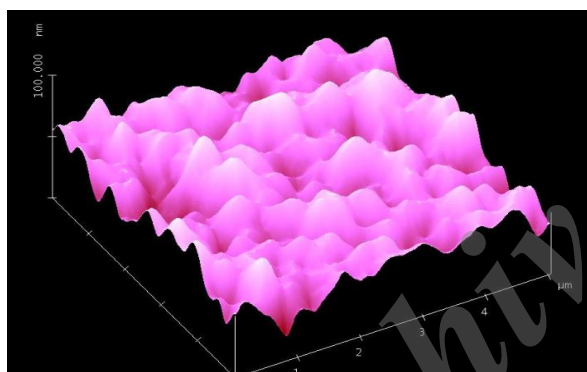
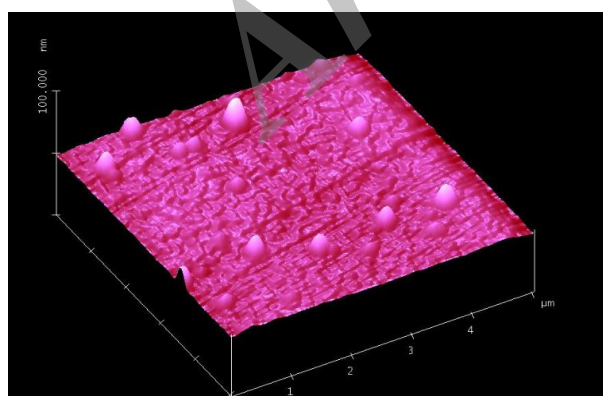
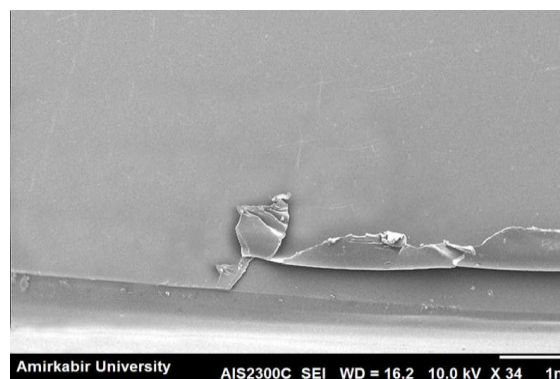
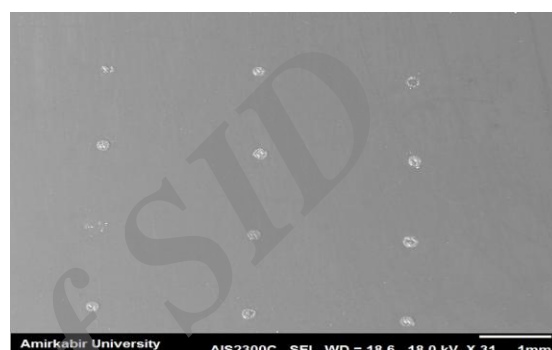
Stability of polymer films in hybridization process

The activated agarose layer grafted to PLL-coated on the glass surface could resist incubation conditions both at 37°C for 6.5 hr and at 50°C for 5 hr. In addition; the stability of agarose layer was examined on the unmodified glass slides. Following the detachment from the slides, it was found that the agarose layer could resist the incubation at 37°C for 3 hr and at 50°C for 1 hr without rehydrating. These slides can be stable during hybridization of biomolecules, such as proteins, peptides and oligonucleotides to microarrays.

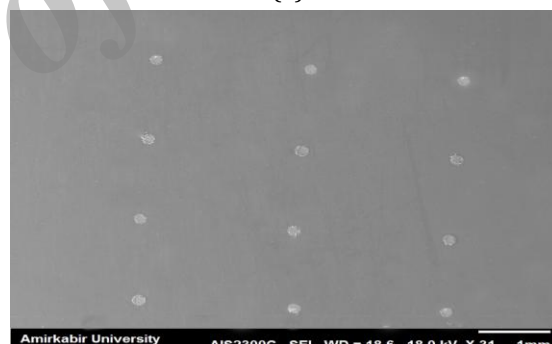
Table 4. Mean reduced elastic modulus and mean hardness of samples

Sample	E_r (GPa)	H (GPa)
Agarose-PLL coated slide	4.613	0.142
Agarose coated slide	5.109	0.16
PLL coated slide	65.545	4.231
Glass slide	72.641	5.454

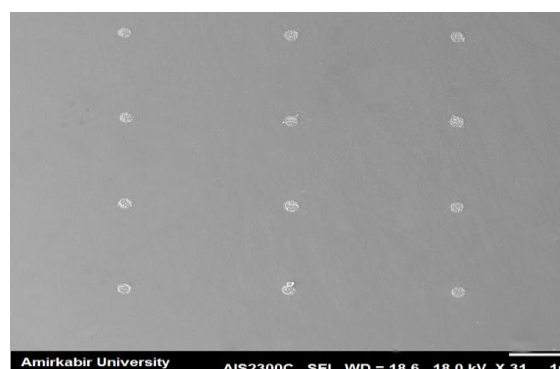
PPL: poly-L-lysine

**Figure 1.** Three dimensional AFM image of agarose-PLL coating. The surface three dimensional structure of agarose on PLL layer is demonstrated**Figure 2.** Three dimensional AFM image of agarose layer on unmodified glass. The three dimensional structure of agarose layer is demonstrated**Figure 3.** Three dimensional AFM image of PLL coating. The PLL coating is a two dimensional layer**Figure 4.** SEM image of agarose layer on the surface of the slides. The agarose coating is parallel to the glass surface

(a)



(b)



(c)

Figure 5. (a) SEM image of spots written on the PLL coated glass slide. The spots were irregular and non-uniform in shape and size. (b) SEM image of spots written on the agarose coated glass slide. (c) SEM image of spots written on the agarose-PLL coated glass slide. The most uniform and regular spots were formed on agarose- PLL coated slide

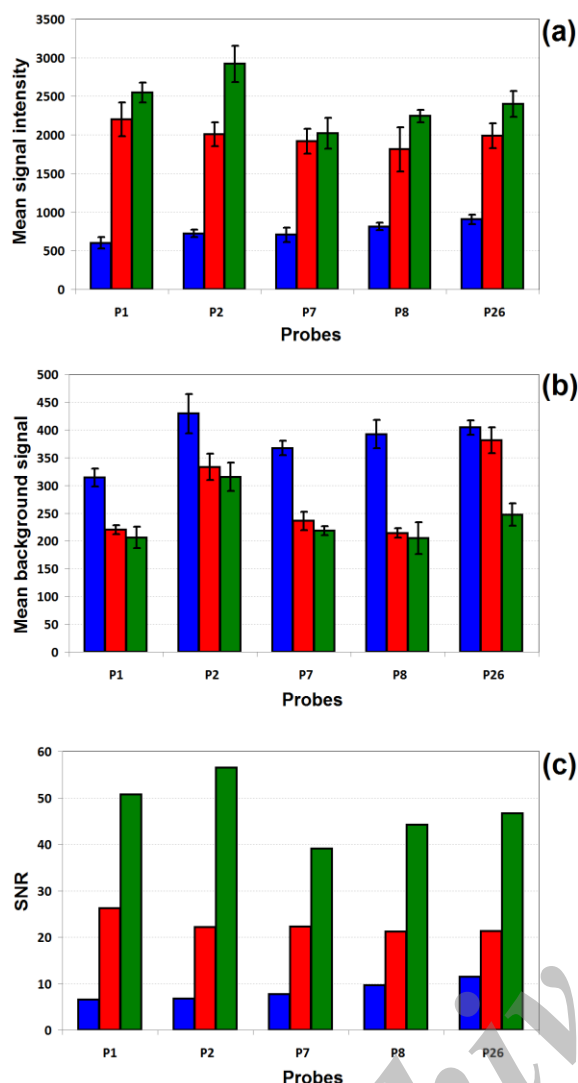


Figure 6. Three different microarray substrate in hybridization reactions. PLL-coated, agarose coated and PLL-agarose coated glass slides were compared in terms of mean signal, mean background signal and signal-to-noise ratio. The highest signal and SNR was obtained for agarose-PLL coated slides. These slides also emitted lowest background signal for each five probes after hybridization

Comparison of different microarray substrate in hybridization reactions

The slides coated with activated agarose and agarose-PLL film showed significant increase in signal and signal-to-noise ratio compared to slides coated with only PLL (Figure 11a, c). Furthermore, background signal from substrate was higher in PLL-coated slides than two other slides coated with agarose. The agarose layer on PLL-coated substrate showed the best hybridization signal (Figure 6b).

The agarose-PLL coated substrates had a 4-fold and 2-fold increase in SNR compared to PLL-coated slides and agarose-coated slides, respectively. These results demonstrated that agarose-PLL coated slides are suitable for analyzing wide concentration range of analytes.

Discussion

The physical methods for modification of glass substrates to immobilize biomolecules provide surfaces with low binding capacity resulting in non-uniform absorption. Consequently, target molecules cannot hybridize efficiently to immobilized probes where an insufficient signal-to-noise ratio may be obtained. Using chemical immobilization, based on covalent crosslinking of surface active groups and functional groups on probe structure, may produce uniform and efficient immobilization of biomolecules on the solid substrate. Agarose gel is a non-toxic and inexpensive polysaccharide which has matrix structure and specific physical and chemical properties, so it has been widely used in biotechnology and life sciences, for example as immobilization substrate in electrophoresis (20).

The results of this study showed that active aldehyde groups in agarose could link to amino groups in probe biomolecules and after UV crosslinking; the covalent binds could be formed. On the other hand, agarose may attach to the surface of glass more strongly if amine groups were provided on the surface. We used a poly-L-lysine coated on the glass surface where PLL was covalently linked to some of the aldehyde groups in activated agarose. PLL is a material used to modify of glass surfaces for preparing microarrays. It can attach strongly to glass surface strongly and provide less interference with background signal. It is also a polymer providing high density of amine groups which can bind more uniformly to agarose. Because of the gel structure of agarose thin film, biomolecules could infiltrate into its microporous structure so increasing its binding capacity. The surface analysis of prepared slides demonstrated that agarose gel was a three-dimensional matrix resulted in increase in surface roughness and decrease in hardness and elastic modulus compared to PLL coated slides and unmodified glass. The hardness and modulus of agarose-PLL layer was lower than agarose coated glass, suggesting the coating of PLL polymer on the glass substrate under the agarose thin film. The vertical distance for agarose-PLL and agarose surface was 35.520 and 33.540 nm, respectively. This parameter could estimate the thickness of thin film layer on the surface. The SEM images of the spots on three different substrates showed spots on agarose-PLL- and agarose-coated glass were more regular in shape and size compared to the spots on PLL coated surface. This observation could be related to the desirable hydrophilicity /hydrophobicity of agarose surface compared to PLL (20).

Long-term stability of agarose layer grafted to PLL was significantly increased compared to agarose on the surface of the other amino-modified glass (19) and agarose on the ordinary glass slides. The agarose on PLL-coated glass sustained 6.5 hr at

37°C and 5 hr at 50°C, whereas agarose film on unmodified glass and agarose layer on the other modified glass was stable 1 hr and 4 hr (19) at 50°C, respectively. This was as a result of covalent binding of agarose to PLL on the glass surface where there is a stronger attachment of agarose to surface than agarose alone.

Effective binding of probes to the surface of agarose-PLL coated and agarose coated substrates reduced noise in hybridization signal compared to PLL coated substrates. In addition, attaching of agarose to PLL coated glass was stronger than that of unmodified glass result in increased signal-to-noise ratio (56.435 compared to 21.235, respectively) and decreased background signal (205 compared to 381, respectively). Consequently, sufficient hybridization was produced on the agarose-PLL coated glass substrates.

In the previous related studies, researchers immobilized amino-modified probes on to agarose-coated substrates. Dufva et al. immobilized 20-25 bp long modified probes by UV irradiation which results in significant increase in hybridization signal (19). In another study, this group immobilized poly (C) 10- poly (T) 10 tagged 17-base oligonucleotide but unmodified probes using UV light on to agarose surface and obtained sufficient discrimination signals (21). In our research, the unmodified longer probes (35-40 bp) were immobilized on agarose surface using UV irradiation. Therefore, despite the connection between few bases of probe end and the gel surface, probes had enough number of nucleotide to retain their specificity for hybridization to targets as ascertained by sufficient signal intensity.

In addition, utilization of PLL coating under agarose layer on glass surface resulted in higher stability and lower background signal in hybridization compared to silane coating under agarose layer (19, 22). This was the reason of low interfere of PLL in fluorescence detection.

Conclusion

In conclusion, PLL/agarose coated substrates can provide sufficient physical characteristics and hybridization reactions as optical microarrays. This technique is inexpensive and simple to use for detection of biomolecules in high-throughput and simultaneous assays.

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