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# Atomic Force Microscopy Surface Nanocharacterization of UV-Irradiated Collagen Thin Films

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*Abstract*—**Collagen, the most abundant protein in mammals, is a basic component of the extracellular matrix and due to its unique properties it is widely used as biomaterial, scaffold and culture substrate for cell and tissue regeneration studies. Due to human skin chronic exposure to sun light and since UV rays are used as sterilizing and cross-linking methods the clarification of the UV light-collagen interactions are very crucial. Moreover, since the majority of the biological reactions occur on surfaces or interfaces the influence of UV light on the surface of collagen-based materials attracts the scientific interest, especially in the biomaterials science. Surface-nanoscale characterization could be performed with Atomic Force Microscopy (AFM), which is a powerful tool and offers quantitative and qualitative information. Its ability of high resolution imaging and non-destructive characterization makes it very attractive for biological samples investigation. The aim of this paper was to determine the surface properties and alterations of collagen thin films after UV-irradiations using AFM techniques. Furthermore, it was aimed to investigate the possible different influence on the surface when the collagen solution or the thin films were irradiated. In this paper topographic AFM images were acquired from thin films, formed from both irradiated and non-irradiated collagen solutions, with spin coating procedure. The results demonstrated that the UV irradiation have different results when it is applied in the collagen solution or in the film after the spin coating methodology. For short irradiation times (<120 min) UV caused only rather small changes in the morphology of the studied films although fluorescence and absorption studies confirmed collagen photodegradation. The surface roughness and topography altered after 3 and 7 hours, respectively, while the fibrous structure was completely destroyed after 15 hours. Surface roughness of the films depends on whether the solution was irradiated or the film and on the time irradiation. The fully clarification of the role of the UV light on collagen thin films will enable the proper design and control of collagen based nanobiomaterials with appropriate and improved surface properties.**

***Keywords-Atomic Force Microscopy, UV, Collagen, Nanocharacterization, Fluorescence***

## I. INTRODUCTION

Collagen is the major fibrillar protein in the extracellular matrix and the most abundant structural protein in mammals. Type I collagen molecules (length ~300 nm, diameter~1,4 nm) consist of three amino acid chains that form rod–shaped triple helices which are assembled to form fibrils [1] and fibrils are aligned laterally to form bundles and fibers. Collagen fibrils are the elementary building blocks in collagen-rich tissues, like skin, and collagen is important for a variety of functions, including tissue scaffolding, cell migration, angiogenesis and tissue repair [2], while its importance in vertebrate biology is crucial [3]. Additionally, collagen contains amino acid sequences that may be recognized by specific receptors and it is shown that cells response to collagen matrix mechanics, topography [4,5] and structure [6]. Collagen is hydrophilic, exhibits negligible cytoxic responses, good hemostatic properties, is readily available and biocompatible [7] and due to these unique properties it is widely used as biomaterial, scaffold and culture substrate for cell and tissue regeneration [8-10].

The investigation of ultraviolent (UV) radiation effects on collagen (from collagen molecules to collagen-based materials) attracts a special interest since UV-collagen interactions are crucial in many research fields. First of all, collagen is a primary target of sun's UV rays, which induce various physical, chemical and physicochemical processes. The absorbance of this deleterious light has been identified as a causative factor for various kinds of skin cancer. On the other hand, there are many cases where the UV radiation is used as a material processing method. UV rays are applied for sterilizing biomedical materials and as a cross-linking method in collagen based biomaterials, so as to improve their mechanical properties [11-13].

Collagen explosion on UV radiation change its mechanical and swelling properties, chemical stability and of course surface properties [14-18]. The surface properties play a very important role in biomaterials since the majority of biological reactions occur on surfaces or interfaces and the quality of the surface features limits their applications. A very useful and powerful technique for studying the surface characteristics is that of Atomic Force Microscopy (AFM), especially when characterization in the nanoscale is demanded. The invention of AFM was a significant improvement in this direction and generally in the field of molecular structures imaging [19]. AFM is a very powerful tool and can offer molecular or

submolecular resolution [12, 20] under different conditions [21]. AFM images of collagen structures, from fibers to separated fibrils or collagen molecules, can be used to obtain qualitative or quantitative information. Properties of films surface, such as the height and the roughness, can be probed without destroying the fibrilllar structure [22].

The purpose of this paper was to determine the surface properties of collagen films after UV-irradiation using AFM techniques. Furthermore, it was aimed to investigate the influence of ultraviolent light when collagen solution or films are irradiated. It must be noticed that the investigation was concentrated on short UV-irradiation exposure times, relevant to those used for sterilization and cross-linking techniques. Thin collagen films were used since their surface can be characterized with AFM [23], which can offer quantitative and qualitative information [24]. What is more, the AFM investigation of the influence that different parameters, like temperature or the used substrates, had on collagen films topography has already been demonstrated [25, 26]. Moreover, this kind of films is appropriate for combining collagen structure and films topography with collagen intrinsic properties like the ability of emitting second harmonic generation signals [27].

## II. Materials and Methods

### A. Collagen Stock Solution

Type I collagen from bovine Achilles tendon (Fluka 27662) was dissolved in acetic acid (CH3COOH 0.5M) in a final concentrations of 8 mg/ml and stored in 4 $^{o}$C for 24h. The solution was then homogenized at 24000 rpm (Homogenizer IKA T18 Basic) and stored in 4 $^{o}$C as the stock solution. Part of the stock solution was irradiated with UV radiation, while both irradiated and non-irradiated collagen solutions were used for the collagen thin film formation.

### B. Collagen Thin Film Formation

The collagen thin films were formed with spin coating and both irradiated and non-irradiated collagen solution were used. Part of the collagen solution (50 μl) was flushed on the substrate, fresh cleaved mica discs (V1, 9.5 diam., 71856-01 Electron Microscopy Science) and spin coated for 40 sec at 6000 rpm (WS-400B-6NPP/LITE Laurell Technologies spin coater).

### C. Samples Ultraviolet Irradiation

Samples (collagen solution and films) were irradiated under air using a GL4 germicidal lamp with a maximum at 254 nm ($UV_{254}$, Sankyo Denki Co. Ltd., Japan). For collagen solution irradiation the experiments were carried out in quartz cuvettes, while the solution was constantly stirred with a micro-submersible magnetic stirred (Model MS-7, TRI-R Instrument, Inc., Rockville Center N.Y.) with the stirring bar placed inside the cuvette. A distance of 3 cm form the light source for various time intervals was used for both collagen solutions and films. The intensity of radiation for this distance was 1813 μW/cm$^2$ (i.e. 0,11 J/(cm$^2$.min) and the dose of incident radiation during the 1 h exposure was 6,6 J. cm$^{-2}$. The intensity of the incident light was measured using a Goldilux$^{TM}$ radiometer-photometer (Model 70234-meter and 70239-probe, Oriel Instruments). All measurements were performed in the same temperature to avoid any influence on the physicochemical properties of collagen.

### D. Spectropic Methods

#### Fluorescence Studies

The emission spectra for collagen solution and films before and after UV irradiation were recorded using Fluorescence Spectrophotometer (Perkin Elmer LS 45 Luminescence Spectrometer). The samples were excited at 240 nm and fluoresce emission maximum was monitored.

#### UV-vissible spectral studies

The UV-Vis absorption spectra for collagen samples before and after irradiation were recorded using a Perkin Elmer Lambda 35 spectrophotometer. For the solution measurements the spectra were recorded using a 1 cm cuvette, while for all the measurements the wavelength range was 230–450 nm with a slit width combination that resulted in a resolution of 2.0 nm. The absorption at 275 nm was tracked since collagen samples have a peak center at this wavelength, which is characteristic of tyrosine. All absorption and fluorescence measurements were carried out at room temperature. The samples were prepared just before measurements.

### E. Atomic Force Microscopy Imaging

AFM images of the collagen films were obtained in the air using a commercial microscope (CP II, Vecco). All the images were obtained at room temperature in contact and intermittent (also named tapping) mode with typical anisotropic AFM probes (MPP-3123 and MPP-1123, respectively). The samples were mounted on AFM metal discs with epoxy glue, while locator grids (Copper finder grid, G2761C, Agar Scientific) were used to map the surface. The topographic AFM images are presented in a color scale which represents the Z height. The image processing and the quantitative measurements were made by using the image analysis software that accompanied the AFM system DI SPMLab NT ver.60.2, IP-Image Processing and Data Analysis ver.2.1.15 (Veeco) and the freeware scanning probe microscopy software WSxM 5.0 dev.2.1 [28]. Images from each sample were taken from several locations and with different image sizes, but only the most representative are illustrated. The surface images, using the scan widths ranging from 3 to 20 μm, with scan rate between 0.5-1 Hz, were acquired at fixed resolution (512 x 512 data points), while some images with scan widths 100 μm where acquired in order to map larger surface areas. The surface roughness was measured for images with scan widths 10 μm and the results are presented in standard deviation. The Root-Mean-Squared Roughness (Rrms) was used, which for a line containing N data points is given by the standard deviation of the data, determined using the standard definition:

$$Rrms = \sqrt{\frac{\sum_{n=1}^{N}(z_n - \bar{z})^2}{N-1}} \quad (1)$$

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where $\bar{z}$ =mean z height. Also the programs can calculate the Rrms for the whole image or regions of interest.

## III. Results

The results and discussion section is divided into two parts. In the first part (A. Irradiation of collagen solution) the results that are discussed concern the irradiation of the collagen solution and then forming the thin collagen films. On the other hand, the second part (B. Irradiation of collagen films) presents the result when the film where first formed and then they were irradiated.

### *A. Irradiation of collagen solution*

Optical measurements were carried out to find out if the photodegration of collagen took place for the used intenisties and irradiation times. Figure 1 shows the fluorescence spectra of the original solution and an AFM topographic image of a film formed from this solution. It can be seen that for excitation with 240 nm there are two emission peaks for native collagen solution. The AFM image demonstrates the fibrillar structure of collagen and that film was consisting of random oriented fibrils. Generally, the roughness of films made of collagen is rather great. Such roughness is a result of molecular architecture of collagen macromolecules.

After irradiation with UV radiation for various time intervals the fluorescence spectra of the collagen solution maintained their characteristics maxima but the emission intensity after irradiation decreased (Figure 2). It was observed that by increasing the time of irradiation, there was a gradual decrease in the emission (Figure 3, Line 1). Complex fluorescent molecules, such as collagen, have more than one fluorophore and any change in their fluorescence spectrum are due to chemical changes in structure of these molecules [29]. Consequently, the state of the collagen molecules influences the emission of fluorescence spectrum.

Furthermore, the intensity of the adsorption spectrum was found to increase on increasing the time of irradiation. The alterations of adsorption at 275 nm is presented at Figure 4. The increase in absorption after irradiation is attributed to the increase in photoproducts formed due to irradiation of the aromatic amino acids, tyrosine and phenylalaline [15].

The irradiated solutions were then used to form collagen thin films. Depending on the time that each solution was irradiated a relevant decrease of the intensity of the fluorescence maxima was also confirmed (Figure 3, Line 2). The difference in fluorescence intensity between solution and film is due to the different type of samples and mainly to the fact that solution measured into 1cm cuvette, while films were formed with spin coating and were extremely thin. Moreover, the absorption spectra demonstrate as in the case of the solution, an increase at 275 nm (Figure 4, Line 2).

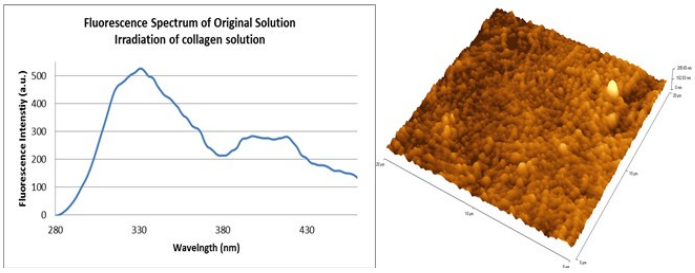


Figure 1. Fluorescence spectrum of the original collagen solution (Left) and AFM topographic images (20x20 μm) of collagen thin film formed from the original solution (Right).

Concerning films surface topography, AFM images demonstrated that after UV irradiation for short irradiation times (10-30 minutes) collagen maintained its fibrous structure (Figure 5) and films' Rrms remained almost constant (Figure 6, Line 1). After 60 minutes of irradiation of the collagen solution, the films that were formed started to have an extremely lower roughness, but after 120 minutes Rrms was again increased. This fact demonstrates that during UV irradiation in solution, denaturation of the collagen triple helix is a reversible process. One the other hand, AFM images showed that for 60 and 120 minuted of irradiaion collagen films maintained their structure and topography (Figure 5, d.).

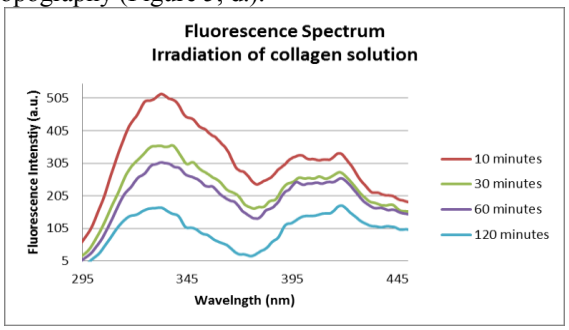


Figure 2. Fluorescence spectra of collagen solution after UV irradiation of 10, 30, 60 and 120 minutes.

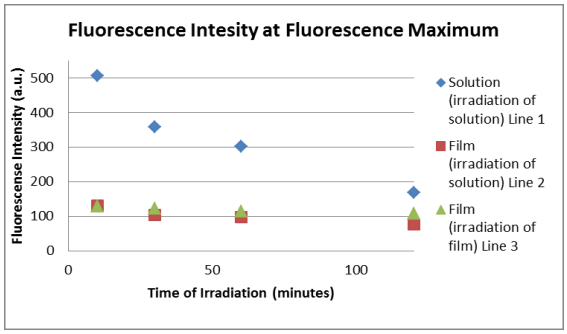


Figure 3. Fluorescence Intensity at fluorescence maximum after UV irradiation of 10, 30, 60 and 120 minutes

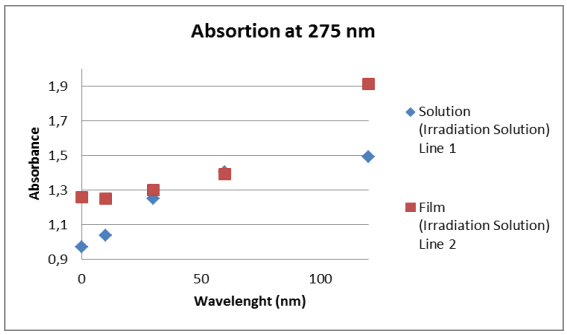


Figure 4. Absorption spectrum of collagen solution after UV irradiation of the solution for 10, 30, 60 and 120 minutes.

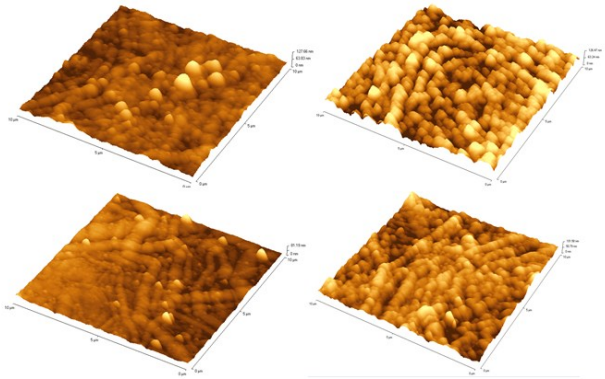

Figure 5. AFM topographic images (10x10 μm) of collagen films formed from collagen solution that was irradiated for 10 (a), 30 (b), 60 (c) and 120 (d) minutes after UV radiation.

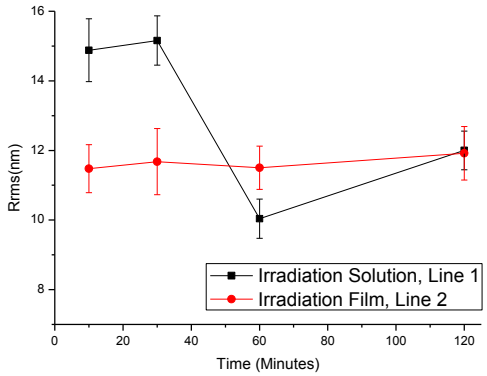


Figure 6. Surface roughness in Rrms of collagen films formed after irradiation of the collagen solution (Line 1, ▪) or the films (Line 2, •) for 0, 10, 30, 60 and 120 minutes UV radiation.

### *B. Irradiation of collagen films*

In order to investigate whether there is a different influence of UV rays on collagen thin films if the irradiation takes place before or after the film formation, the same experiments were repeated for films that were first formed and then irradiated.

The fluorescence spectra present similar behavior after UV irradiation with the emission intensity to decrease by increasing the time of irradiation (Figure 3, Line 3). The fluorescence intensity at fluorescence maximum presented exact the same decrease as in the case of the film that was formed by irradiated solution (Figure 3, Line 2).

For investigating the influence of UV radiation with AFM, the same film was used and was imaged immediately after each set of irradiation. This fact and the use of locator grid enabled the imaging of exact the same area of the film so as to have more accurate results. The images demonstrated that for short irradiation times (10-60 minutes) the film morphology did not present any significant alteration (Figure 7). The surface roughness was also kept almost constant (Figure 6, Line 2), although by comparing the surface roughness with that of films that were formed by irradiated solution, it can be seen that the second ones have higher roughness. This demonstrates that the order of UV processes does not influence the structure of collagen film but affect the surface roughness of the formed films.

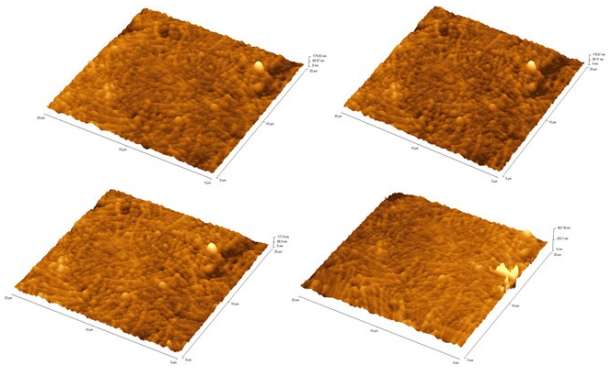

Figure 7. AFM topographic images (20x20 μm) of collagen film that was irradiated for 10 (a), 30 (b), 60 (c) and 120 (d) minutes with UV radiation.

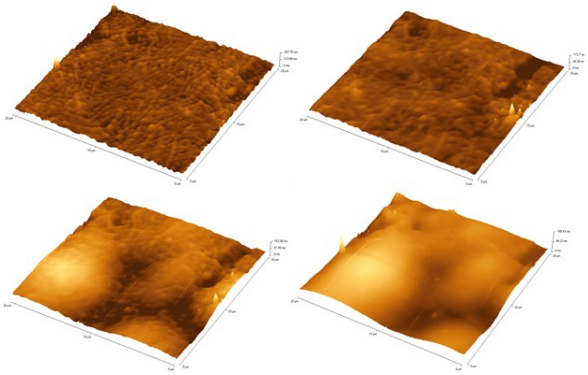

Figure 8. AFM topographic images (20x20μm) of collagen film that was irradiated for 3 (a), 7 (b), 11 (c) and 15 (d) hours with UV radiation.

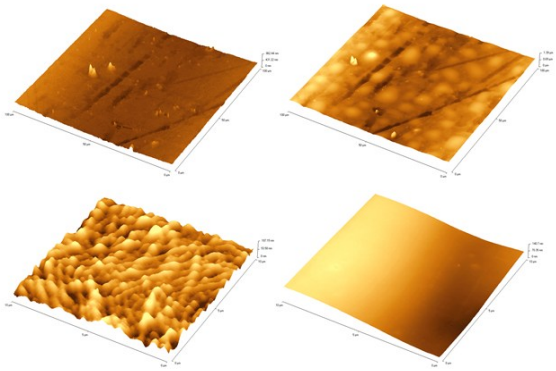

Figure 9. AFM topographic images (100x100μm first row, 10x10 μm second order) for 3 and 15 hours UV irradiation, first and second column respectively.

In order to investigate whether UV rays can alter surface morphology for higher irradiation times, the same film was exposure to UV radiation for 3, 7, 11 and 15 hours. The images in Figure 8 demonstrate that the film for 3 and 7 hours of irradiation did not present any significant alteration on their surface. After seven hours it was only observed that surface roughness was significantly reduced to 6,73 nm (with a standard deviation of 0, 36 nm).

After 11 hours of irradiation, films start to swell and expand by destroying film topography and deforming collagen structure (Figure 8 and 9). In large scale images film surface present a bubble-like pattern, while in small scale images it can be confirmed that collagen fibril structure was destroyed. In

case of fibrous collagen, other than collagen solution, denaturation of the triple helix is an irreversible rate process [30]. The surface roughness was dramatically altered and presents both high and low values depending on the specific surface area and on the level of the surface expansion and collagen destruction.

## IV. Discussion

Since the results demonstrated that the emission intensity of collagen decrease by increasing the duration of UV irradiation, it is provided evidence that conformational changes around the aromatic amino acid residues or structural changes of the residues themselves take place [15]. Furthermore, the increase absorption at 275 nm is a result of the increase in the number of photoproducts and shows that the photodegration of collagen results in the scission of peptide linkage and and the subsequent tyrosine formation from phenylalaline, which is one of the main constituents of collagen by an oxidative reaction. That is why the formation of tyrosine is said to be an initial step for the photo-aging of skin. What is more, it is believed that in acid soluble collagen, as in our case, under UV radiation free radicals appear which result in photodegration of the collagen macromolecule [17].

Concerning the irradiation of collagen films, the results showed that for short irradiation times the UV process affects the surface roughness but does not influence the collagen structure. Consequently, in cases that collagen thin films are aimed to be used as substrates for culturing cells and taking into account that surface roughness influence the cells behavior, the UV irradiation can be used not only as a sterilizing and cross-linking method but also as a process to control surface roughness. For higher irradiation times the UV alters dramatically both structure and surface roughness, indicating that only short irradiation times should be used for biomedical applications so as the collagen to maintain its native structure.

In previous studies it was demonstrated that by using similar radiation energies at the same wavelength, UV irradiation after 12 hours caused decrease of surface roughness of pure collagen (2.6->2.1 nm) and PVA(poly-vinyl alcohol)-collagen films [18]. The decrease of surface roughness by UV irradiation of similar energies, was also confirmed in collagen-poly-vinyl pyrrolidone( PVP) films [31]. The decrease of the surface roughness that was observed in this paper was higher since the original collagen solution that was used, had significantly higher concentration. The damage of collagen structure has been also shown in previous AFM and contact angle studies of polymer films containing small amount of collagen where after UV irradiation the surface free energy was altered indicating photooxidation and increase of the polarity of the surface [7]. It was also demonstrated an increase of surface polarity after irradiation of pure collagen samples, which suggests that efficient photooxidation took place on the surface. What is more, it has also been shown that UV-C irradiation damaged and led to lower thermal stability not only collagen but also model collagen peptides [16]. Furthermore, it has been shown that physiologically attainable doses (0.02-1 J/cm$^2$) of direct UV radiation had no detectable effect on type I collagen monomers [32]. The results presented in the paper have shown that UV irradiation induces the phooxidation of collagen films, but the surface topography is altered after a significant time of irradiation. Consequently for short irradiation times, collagen films maintain their surface properties and UV irradiation process can be used for cross-linking and sterilization purposes.

## V. Conclusion

From the results of this paper it was demonstrated that the UV irradiation procedure had different effects when it was applied in collagen solution or on the film after the spin coating methodology. The absorption and fluorescence spectra demonstrated that for the used intensities and for short irradiation times (<120min) the physical phenomenon of collagen photodegration took place on both the irradiated films and solutions. On the other hand, the AFM investigation showed that UV irradiation caused only rather small changes in the morphology of the studied films. For these irradiation times the major influence was found to be on the surface roughness. While films that were first form and then irradiated presented a constant Rrms, those films that were formed from irradiated solution had higher roughness which changed with irradiation time. The change in Rrms was not linearly depended on the time of irradiation and presented fluctuations, which shows that the collagen denaturation in solution is a reversible process. In the case of the collagen films the surface roughness started changing after 3h, while after 7h topography alterations were obvious. After 11h the film surface was significantly expanded and swelled and after 15h the collagen fibrous structure was completely destroyed. As UV-collagen interactions are crucial, their clarification will enable the proper design and control of collagen biomaterials with appropriate and improved surface properties and the results of this paper offer information toward this direction.


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