Enamel Based Composite Layers Deposited on Titanium Substrate with Antifungal Activity


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The goal of this paper was to investigate the antifungal activity of Enamel layers deposited on titanium substrate (Ti-Enamel) and Enamel layers deposited on titanium substrate previously coated with a vinyl polydimethylsiloxane layer (Ti-PDMS-Enamel). The physicochemical properties were also investigated. The Candida albicans biofilm development on the obtained layers was examined after 24 h, 48 h, and 72 h by confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM) after ethidium bromide staining. A significant inhibition of the fungal adherence and biofilm development was observed on Ti-Enamel layers. The antifungal results demonstrated that the use of new Ti-Enamel composite layers could represent a promising perspective for the prevention of fungal biofilms associated implant infections.

1. Introduction

Biomaterials [1–6] can be considered the “building blocks” of modern medicine, due to their multiple applications, either for the fabrication of medical devices (heart valves prostheses, artificial hip joints, dental implants, intraocular lenses, left ventricular assist devices, wound dressings, artificial skin, degradable sutures, scaffolds for cell and tissue transplants, arterial stents, etc.) or for diagnostic and theranostic platforms, biomolecules isolation, purification, characterization, and so forth [2]. An important aspect of the medical application as coatings for orthopedic implants consists in improving the biocompatibility and osteointegration of prostheses [7–10]. The need for materials that can improve the bonding ability of an implant to bone tissue is at high demand because this represents a fundamental requirement for a successful implant in clinical use [11, 12]. Therefore, the search for the optimum combination of implant and coating is crucial when developing an implantable device. The most used metal in the field of orthopedic implants is titanium,
due to the optimal combination of a good biocompatibility and of suitable mechanical properties. The coating used for improving the inert titanium biocompatibility is usually a bioactive ceramic, such as hydroxyapatite (HAp) or other similar bioactive materials [13]. Even though there have been remarkable progresses made in the area of HAp biomedical applications, there are still very important issues that need to be addressed. One of the most important ones is the apparition of postoperative infections and the difficulty in treating them with conventional antibiotics [14–17]. The emergence of antibiotic resistant pathogens represents a major health issue and it has been reported that more than 70% of the bacterial infections cannot be treated with conventional antibiotics [18–20]. In the last 30 years infections caused by fungal strains have been notably increasing in a wide range of patients from the immunocompromised individuals to the ones with mild health problems. Considered to be one of the most dangerous fungal organisms, the Candida strains are responsible for the occurrence of the most usually acquired hospital related infections [21]. The most representative strain of Candida, that is, Candida albicans, can be the cause of vaginal, oral, and systemic infections in hospitalized patients, causing severe and sometimes deadly affections. Recent estimates of the National Institutes of Health have reported that more than 80% of the microbial fungal infections are caused by biofilm growing microorganisms [21–24]. Candida albicans biofilms are highly complex structures formed by multiple cell types embedded in an extracellular matrix [25]. More than that, C. albicans is the most frequent microbial strain isolated from medical devices such as catheters, pacemakers, mechanical heart valves, joint prostheses, contact lenses, and dentures associated with device-related infections. Statistical data from United States show that each year more than five million catheters are placed and a biofilm infection appears in more than 50% of the cases [26]. Moreover, the resistance of these fungal biofilms to current antibiotics has led to considerable efforts in treating the infection often leading to a combination between a supplementary surgery to remove the infected device and the administration of high doses of antibiotics. Most of the times these procedures are dangerous to the patient, they can be costly, or they could be impossible to perform due to the precarious condition of the patient [27].

In this context, a great deal of attention was aimed towards the improvement of the antimicrobial properties of the materials used for the fabrication of medical devices. The ability to control bacterial biofilm development would be advantageous and helpful in eliminating the risk of biofilm implant associated infection in patients. Therefore, there have been several studies reporting materials with antimicrobial properties that could also be used as coatings for medical metal devices. The most investigated materials related to the apparition of infections caused by microbial biofilm formation are in dentistry. Several studies have reported bioactive restorative materials which possess antimicrobial properties [27–30]. Montanaro et al. [31] have emphasized in their study that different microbial strains adhesion on dental restorative materials could be influenced not only by the particular surface chemistry of the material but also by the different roughness of the material surface. Several studies depict Enamel as being the obvious choice when aiming at achieving both biocompatibility and antimicrobial properties. Enamel is found in the outer hard tissue layer of tooth crowns, is the hardest bioceramic that can be found in the vertebrate body, and has a unique complex structure. This composite contains hydroxyapatite crystals, water, and an organic matrix. Even though the available data for Enamel (mainly composed of hydroxyapatite which is well known for its biocompatible properties) is quite limited, the results regarding its antimicrobial properties presented by us suggest that it can be considered as a coating material for medical implantable devices. Nevertheless, the combination of metal mechanical properties and of a bioactive and antimicrobial coating is not sufficient in order to achieve good results in the medical field. In clinical use, imperfections such as weak adhesion of the coating on the metal substrate or low scratch resistance could significantly narrow the practical applications [32, 33]. In their effort to solve these impediments, during the last decades researchers worldwide proposed both the use of various deposition techniques and the use of a polymer with the scope of immobilizing the structure. For that purpose, in our previous studies [34], we proposed the use of a polydimethylsiloxane (PDMS) as an interlayer for a silver doped hydroxyapatite (Ag:HAp) coating. The studies conducted by Popa et al. [34] revealed that the PDMS layer acted as a matrix for the incorporation of the Ag:HAp, thus creating a crystalline Ag:HAp-PDMS composite layer.

The goal of the present research was the fabrication, characterization, and in vitro evaluation of Candida albicans development on Enamel, PDMS, and PDMS-Enamel layers deposited on titanium substrate.

2. Experimental Method

2.1. Deposition of Enamel Layer on Commercially Pure Ti Disks (Ti-Enamel). The Enamel layer has been deposited on the titanium substrate using a thermal evaporation technique [34].

2.2. Deposition of Enamel Based Solid Layers on Titanium Substrates Previously Coated with a PDMS Layer (Ti-PDMS-Enamel). Firstly, the PDMS layer was deposited, starting from vinyl terminated polydimethylsiloxane (Ti-PDMS) liquid precursors, on Ti substrate in atmospheric air pressure corona discharges. The entire deposition procedure was depicted in detail in [35, 38]. After that, the Enamel layer was deposited on the titanium substrate previously coated with PDMS using a thermal evaporation technique according to a previously reported paper [34].

3. Characterization Methods

3.1. Scanning Electron Microscopy (SEM). The morphological analysis of the Enamel based coating surface in the presence and absence of a PDMS layer has been investigated by scanning electron microscopy (SEM) using a FEI Inspect S scanning electron microscope in both high- and low-vacuum modes.
3.2. Energy Dispersive X-Ray Spectroscopy (EDS). Elemental compositional analysis was done using energy dispersive X-ray spectroscopy (EDS). All spectra were acquired with an EDS Inc. SiLi detector attached inside the scanning electron microscope. The measurements were performed at 10 keV in order to avoid the substantial presence of metal atoms in the spectrum.

3.3. Fourier Transform Infrared Spectroscopy (FT-IR). Ti-Enamel and Ti-PDMS-Enamel layers were investigated by FTIR spectroscopy using a SPI100 IR Perkin Elmer spectrometer equipped with a variable angle specular reflectance accessory. The spectra were carried out for an angle of reflection of 30°.

3.4. Glow Discharge Optical Emission Spectroscopy. The elemental depth profile analysis of the Ti-Enamel and Ti-PDMS-Enamel layers was performed by Glow Discharge Optical Emission Spectroscopy. The experimental conditions used for the operation of the GD Profiler (Horiba Company) were 650 Pa, 35 W RF power impulse mode at 1 kHz, and a duty cycle of 0.25.

3.5. Confocal Laser Scanning Microscopy (CLSM). Confocal laser scanning microscopy (CLSM) was performed to study the Candida albicans development on different substrates. The images were obtained using a Leica TCS SP (Leica Microsystems, Germany) confocal laser scanning microscope. The samples were stained with ethidium bromide (5 µg/mL) for five minutes before microscopic examination by CLSM. The images were collected using a Leica HCX PL FLUORITE 40x/0.75 NA dry objective (Leica Microsystems, Germany) with excitation with the Ar ion laser at 488 nm and an emission between 580 and 660 nm. Under this imaging configuration, typical confocal resolution is of the order of 250 nm in the lateral direction and 600 nm in the axial direction.

Additional images were obtained using a Nikon C2 confocal laser scanning microscope. The samples were stained with ethidium bromide (5 µg/mL) for five minutes before microscopic examination by CLSM. The images were collected using a Nikon CFI Super Plan Fluor ELWD 40x/0.6 NA with excitation at 488 nm and an emission between 580 and 660 nm.

3.6. Antifungal Assay. In order to assess the antifungal activity of the Ti-Enamel, Ti-PDMS-Enamel, and Ti-PDMS layers, Candida albicans ATCC 26790 strain was used.

3.7. Fungal Biofilm Development on the Ti-Enamel, Ti-PDMS, and Ti-PDMS-Enamel Layers. The C. albicans ATCC 26790 strain was purchased from American Type Culture Collection (ATCC, US). The monospecific biofilm development on the obtained substrata was performed using sterile 6-well plates (Nunc). Sterile coated and reference specimens were added in a plate well in 2 mL of LB broth inoculated with ~105-106 colony forming units (CFU)/mL of fungal suspension. The samples were allowed to incubate at 37°C for three time periods (24 h, 48 h, and 72 h) to assess the temporal dynamics of biofilms developed in the presence of tested samples. For the qualitative evaluation of the fungal biofilm development, after incubation, the samples were carefully washed with sterile saline buffer to remove any unattached microbial cells and then fixed with cold methanol and stained with ethidium bromide (5 µg/mL solution in distilled water). The colonized specimens were submerged in the staining solution and incubated in dark, for five minutes, at room temperature. After incubation, the excess of the staining solution was removed using a filter paper and the specimens were examined immediately. The samples were visualized in reflection and transmission mode by using a Leica microscope (TCS-SP CSLM model), equipped with PL FLUOTAR (40x NA 0.7, electronic zoom 1), and a He-Ne laser tuned on 633 nm wavelength. A lateral resolution of about 600 nm was achieved. The Leica software was used for examining the surface topography [39].

In order to examine the adherence of viable cells on the substrates surface, the samples were immersed in free culture medium inoculated with standard fungal suspension, incubated in static conditions for 2 hours, and then examined using a Nikon C2 confocal laser scanning microscope. At the end of the incubation period, the substrata were removed from the suspension, gently washed with PBS to remove the unattached fungal cells, and then stained with ethidium bromide (5 µg/mL) for five minutes and examined immediately in order to highlight the adherence of viable fungal cells on the substrates surface.

3.8. Quantitative Evaluation of Fungal Biofilm Development

3.8.1. Total Biofilm Assay. The monospecific biofilm was grown on the obtained sample in sterile 6-well plates (Nunc) containing 2 mL of LB broth inoculated with ~105-106 colony forming units (CFU)/mL of fungal suspension. The samples were allowed to incubate at 37°C for three time periods (24 h, 48 h, and 72 h) to assess the temporal dynamics of biofilms developed in the presence of tested samples. After incubation, the samples were carefully washed with sterile saline buffer in order to remove the unattached microbial cells and then immersed in 1 mL sterile saline buffer in Eppendorf tubes to get biofilm detachment by vigorous vortexing. The density of the recovered suspension was determined spectrophotometrically, by measuring the absorbance at 600 nm.

3.8.2. Viable Counts Assay. The sterile substrata were added in a sterile saline inoculated with ~105-106 CFU/mL of microbial suspensions. The samples were incubated under stirring at 37°C for 72 h. The number of fungal viable cells developed in the presence of the samples was assessed using 10 µL of fungal suspension at 24 h, 48 h, and 72 h. For this purpose the fungal suspensions were further diluted and 10 µL of each serial dilution was plated in duplicate on LB agar. After 24 h of incubation at 37°C, viable cell counts were performed and the number of CFU/mL for each sample was evaluated.

3.9. Image Analysis. The CLSM images acquired during the experimental session were processed using ImageJ software.
Figure 1: SEM images of Ti-Enamel (a) and Ti-PDMS-Enamel (b) layers and Ti substrate (c).

Figure 2: EDS spectrum of Ti-Enamel (a) and Ti-PDMS-Enamel (b) layers.

The Candida albicans biofilms developed on the surface of the layers were investigated using a three-dimensional (3D) model. The 3D reconstruction of the CLSM images was used for the C. albicans biofilm thickness estimation.

3.10. Statistical Analysis. All the experiments regarding the antifungal properties of the layers were done in triplicate and repeated three times. The biofilm development was estimated by plotting the mean absorbance values of C. albicans ATCC 26790 biofilm cells developed on the samples. The statistical analysis was conducted in agreement with other previous studies on surface and biological evaluation of hydroxyapatite-based coatings on titanium deposited by different techniques presented by Massaro et al. [40]. Furthermore, the data image analysis was carried out using the Student t-test and the values of \( p < 0.05 \) were accepted as significant.

4. Results and Discussions

The surface morphology of the Ti substrate, Ti-Enamel, and Ti-PDMS-Enamel layers was examined by scanning electron microscopy (SEM).

The features of the investigated samples are presented in Figures 1(a)–1(c). It can be observed that the layers are homogeneous with no cracks (Figures 1(a) and 1(b)). Figure 1(b) suggests that the presence of the polymer layer on the Ti substrate during the Enamel deposition process determines a more pronounced granulation of the Enamel.

The EDS spectra of Ti-Enamel and Ti-PDMS-Enamel coatings are presented in Figures 2(a) and 2(b). In both spectra the elements (Si, Ti, P, Ca, Ba, Na, K, and Al) contained by the studied layers can be identified. Moreover, it could be observed that the peaks associated with Si and O elements are more intense in the case of PDMS-Enamel coatings deposited on Ti substrate compared to those associated with the Enamel layer.
The main functional IR groups present in the structure of Ti-Enamel and Ti-PDMS-Enamel layers are listed in Table 1.

![Figure 3: IR spectra of Ti-Enamel (a) and Ti-PDMS-Enamel (b) layers.](image)

The molecular structure of the Enamel and PDMS-Enamel layers uniformly deposited on mirror-like surfaces of Ti substrates has been investigated by reflectance FTIR spectroscopy (Figure 2) [34, 35, 38]. As it is already known, Enamel powders have an apatite-like structure and the main vibrational IR bands are attributed to PO₄³⁻, OH⁻, and CO₃²⁻ vibrational groups [37, 41–43].

The specific vibrations of these chemical groups were identified for Ti-Enamel layers presented in Figure 3(a) and summarized in Table 1. The IR bands from 1042 cm⁻¹ and 1090 cm⁻¹ are typical for apatite phosphorus-oxygen absorption bands in Enamel powders.

The observation of these bands in an Enamel based coating certifies that the deposition process was a success. The 640 cm⁻¹ vibrational band was previously observed in synthetic hydroxyapatite and Enamel powders and was attributed to hydroxyl ions vibrations [37]. In the IR spectrum of the Ti-Enamel layer, the Si-O IR specific bands were also identified. The Si-O, Si-O-Si, and Si-CH₃ specific bonds of PDMS layers [36] are also present in the IR spectrum of Ti-PDMS-Enamel presented in Figure 3(b). The chemical groups found in the Ti-PDMS-Enamel spectrum were summarized in Table 1.

The elemental depth profiles of the Ti-Enamel and Ti-PDMS-Enamel layers were investigated by Glow Discharge Optical Emission Spectrometry (GDOES) in order to analyze the distribution of Ca, P, O, C, Si, K, Na, Ba, Al, and H atoms at the Enamel based layer in the presence or absence of the PDMS layer. As working principle of this instrument, a few millimetres’ squared area from the investigated sample is sputtered in a pulsed RF Ar plasma. Consecutively, the intensity of the light emitted by the sputtered atoms excited in the plasma is acquired and recorded in real time. This provides as the investigated sample is sputtered from its surface down to the substrate as a function of sputtering time. The sputtering rate has specific values for each material, varying during the elemental depth profiling measurement (acquisition). The depth profile curves of the Ca, P, O, C, Si, K, Na, Ba, Al, and H atoms in the bulk and at the surface of the Ti-Enamel layer are presented in Figure 4(a).

Even if the elements specific to the Enamel have similar profiles to the Ti-Enamel and Ti-PDMS-Enamel layer, respectively (Figure 4(b)), their intensities and widths are different. At the same time, in Figure 4(b), we could not identify a sharp delimitation between the elements present in the Ti-Enamel and Ti-PDMS-Enamel layer. Different reasons like roughness...
of the layer/substrate interface as the signals recorded by the GD Profiler are averaged over the investigated zone, crater bottom flatness, or a composite material could determine such behavior. As the Ti substrate is a mirror-like surface and the operating conditions were chosen for providing a flat crater bottom, the GD depth profiles from Figure 4(b) seem to indicate the formation of a composite material. As the intensity of the Si depth profile in the Ti-PDMS-Enamel layer decreases, the Ca, P, O, C, K, Na, Ba, Al, and H depth profile intensities decrease, indicating the incorporation of the Ti-Enamel components into the PDMS layer. The increase of the Ti depth profile curve simultaneously with the decrease of all the elements contained by the investigated layer indicates the substrate interface [34].

The development of *C. albicans* biofilm on medical devices is following three stages: (i) the adherence of yeast cells to the device surface (0–11 h); (ii) the secretion of extracellular matrix and the switch from yeast to hyphal forms (12–30 h); and (iii) the development of a three-dimensional, dense biofilm containing both yeast and hyphal forms (38–72 h) [25, 44]. Due to the important implications of *Candida albicans* strains in the pathology of prosthetic devices related infections, aggravated by the emergence of resistant fungal strains, using new coatings for titanium implants able to inhibit the fungal adherence could represent a promising perspective for the prevention of biofilm associated implant infections.

The quantitative results regarding the *in vitro* biofilm formation by *C. albicans* 26790 on the surface of Ti-Enamel, Ti-PDMS-Enamel, and Ti-PDMS layers as determined by measuring the density of the microbial suspension recovered from the biofilms adhered on the tested samples are represented in Figure 5.

The quantitative results showed that the fungal biofilm development was inhibited both in early phase of adherence, quantified after 24 h, and in the development and maturation stages, quantified after 48 h and 72 h of incubation, respectively. The absorbance of microbial suspensions recovered from the biofilms developed on the Ti-Enamel layers was in all cases significantly reduced as compared to that of biofilm cells developed on the Ti-PDMS layers.

Furthermore, the viable cell count (VCC) assay confirmed the results of the total biofilm assay, as revealed by the significant decrease of the VCCs recovered from the Ti-PDMS-Enamel as compared to Ti-PDMS after 24 h of incubation.
The CLSM technique was used for the investigation of C. albicans ATCC 26790 biofilm cells developed on the Ti-Enamel, Ti-PDMS-Enamel, and Ti-PDMS layers. The 3D reconstruction was used to determine the average thickness of the C. albicans biofilms developed on the surface of Ti-Enamel, Ti-PDMS-Enamel, and Ti-PDMS layers. The biofilm thickness was determined to be around 60 μm in the case of Ti-PDMS layers and steadily decreased with time for the Ti-PDMS-Enamel and Ti-Enamel layers reaching a minimum of approximately 30 μm for the Ti-Enamel layers at 72 h. The biofilm thickness obtained from three different experiments is presented in Table 2.

The results obtained for the biofilm thickness are in agreement with other studies which reported C. albicans biofilm formation on different kind of substrates [47]. In their studies, Chandra et al. reported a biofilm thickness of 25 μm formed on denture acrylic in vitro [25]. However, Ramage et al. reported a biofilm thickness exceedingly greater of 70 μm [48] and in their studies, Andes et al. [47] found that many areas of the biofilm in their in vivo catheter model exceeded 100 μm. Depending on the time of incubation and the substrate used, studies [25, 47, 48] have reported an average thickness of a C. albicans biofilm ranging from 15 to 100 μm. The precedent studies suggested that the difference in thickness may be related to the time allowed for incubation or adherence.

More than that, previous studies [25, 49] have shown that fungal biofilms grown on denture and catheter material become resistant to traditional antifungal agents. Also, it is well known that, similar to their bacterial counterparts, C. albicans biofilms are highly resistant to antimicrobials. Although drug resistance has been shown in C. albicans [25, 49] and bacterial biofilms [50, 51], this is a complex study which reports results regarding the antifungal activity of Ti-Enamel layers against C. albicans biofilms.

In good agreement with the previous studies presented above, our results suggested that the thickness of the biofilm is strongly dependent on the substrate and also on the incubation time. The results regarding the thickness of the C. albicans biofilms determined using a 3D reconstruction model of the CLSM images were also in accordance with the existing studies.
Furthermore, our results regarding the use of these samples in different biomedical fields such as dental or orthopedic implants are in good agreement with previous studies on nanocomposite hydroxyapatite conducted by Baker et al. and Groza et al. [52, 53].

The present studies provide the new perspectives of Enamel and PDMS-Enamel layers on Ti substrate for potential use in medical field. Moreover, these studies are part of the pharmaceutical and medical industry effort to develop new materials with antimicrobial properties. Future studies regarding genetic linkage of Enamel and PDMS-Enamel layers resistance genes and antibiotic resistance genes will be conducted.

5. Conclusions

Enamel and PDMS-Enamel layers were deposited on Ti substrates using a thermal evaporation technique. The morphology of these layers was investigated by scanning electron microscopy. SEM micrographs revealed that the presence of a polymer interlayer on the Ti substrate during Enamel deposition led to a pronounced granulation of the deposited
powder. The elemental composition of the Ti-Enamel and Ti-PDMS-Enamel layers was investigated using EDS and GDOES analysis. The EDS results confirmed the presence of the constituent elements of both types of layers (Si, Ti, P, Ca, Ba, Na, K, and Al). More than that, the GDOES depth profiles of the Ti-PDMS-Enamel indicated the formation of a composite material. FTIR spectroscopy used to investigate the chemical structure of the Ti-Enamel and Ti-PDMS-Enamel layers revealed the presence of the Enamel specific bands in both samples. The presence of these bands in the FTIR spectra confirmed that the deposition process was successful. *C. albicans* biofilm development on the Ti-PDMS,
Figure 9: CLSM examination for viable *C. albicans* biofilm cells stained with ethidium bromide developed on the Ti-Enamel, Ti-PDMS-Enamel, and Ti-PDMS layers.

Figure 10: Three-dimensional reconstruction of *C. albicans* biofilms developed on different substrates at various time intervals using confocal microscopy. (a) *C. albicans* biofilm development on Ti-Enamel layer after 24 h; (b) *C. albicans* biofilm development on Ti-Enamel layer after 48 h; (c) *C. albicans* development on Ti-Enamel composite layer after 72 h; (d) *C. albicans* development on Ti-PDMS-Enamel layer after 24 h; (e) *C. albicans* development on Ti-PDMS-Enamel layer after 48 h; (f) *C. albicans* development on Ti-PDMS-Enamel layer after 72 h; (g) *C. albicans* development on Ti-PDMS layer after 24 h; (h) *C. albicans* development on Ti-PDMS layer after 48 h; (i) *C. albicans* development on Ti-PDMS layer after 72 h.

Ti-PDMS-Enamel, and Ti-Enamel layers was analyzed by SEM and CLSM at various time intervals (24, 48, and 72 h). SEM and CLSM observations revealed that *C. albicans* biofilm development was time dependent. The present studies evidenced that Enamel layers had a fungicidal effect against *C. albicans* biofilm formation. The qualitative observations regarding the antifungal activity exhibited by the Ti-Enamel layers were confirmed by quantitative investigations, showing that the fungal biofilm development was inhibited at all tested time intervals by Ti-Enamel layers. Moreover, the viable cell count (VCC) confirmed the qualitative and quantitative results and revealed a significant decrease of viable cells in the...
case of Ti-Enamel, comparative to Ti-PDMS-Enamel and Ti-PDMS layers after 24 h of incubation. The C. albicans biofilms exhibited a thickness of 60 μm for the Ti-PDMS layers, which has decreased at around 30 μm for the Ti-Enamel layers. The biofilm formation is strongly dependent on the incubation time as well as the substrate used.

Because fungal infections come to be an increasingly alarming problem worldwide, the need for new antifungal agents represents a priority. Thus, the results presented in this paper could lead to a better understanding of the fungal development behavior which might add diversity and useful information to the currently exceedingly limited research data. Furthermore, the data obtained may lead to the conclusion that Enamel and Enamel based layers could be considered in the development of new antymycotic agents.

### Competing Interests
The authors declare that there is no conflict of interests regarding the publication of this paper.

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