Assessment of potential cancer protection of cosmetic products of agro-food origin by Zeolite Scaffolds
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Abstract—In this work, three different pure inorganic zeolite membranes and three hybrid PLA-containing Mixed Matrix Membranes (MMMs) were fabricated and compared with each other in virtue of their ability to interact with cells for in vitro test applications. Additionally, we report their performances in cell experiments of novel olive oil-containing cosmetics by using two cell lines with (MCF-10A) epithelial and (MCF-7) epithelial-like cancer characteristics. Our in vitro results revealed that all zeolite scaffolds permit to obtain higher cell densities compared to pure polymeric scaffolds. We also describe that epithelial cells preferentially adhere on pure membranes according to decreasing order: Linde type L>Fe-S-1>Fe-ZSM-5. Moreover, we evidenced an opposite behavior of cancer cells that prefer to adhere and growth on MMM scaffolds. This study shows that the anticancer characteristics of novel cosmetics of natural origin can be easily determined and emphasized using zeolite membranes.

Keywords—Cell culture, Cosmetics, PZC, Scaffolds, Zeolite membranes.

I. INTRODUCTION

Various studies reported in the literature seem to indicate that olive oil can be directly used on the skin and locally applied in the forms of creams or salves because the topical use of olive oil alone or as an ingredient in cosmetics shows therapeutic effects (anti-inflammatory, anti-neoplastic and anti-aging) [1-3]. The possible carcinogenicity of each component of a novel cosmetic product can represent an alarming characteristic, which frequently represents the biggest barrier to its commercialization. In the past, many tests on cancer induction from novel cosmetics were carried out using animals. Since 2004, in vivo tests are prohibited in the European Union and the commercialization of cosmetics containing component tested on animals was forbidden and a ban extension until March 2013 was agreed [4]. Today it is a pressing need to design novel scaffolds that have high cellular densities, durability, reproducibility and low degradation kinetic in culture media characteristics due to intrinsic properties of materials. Recently, many synthetic zeolites have been shown to be biocompatible materials [5,6] that allow the growth of different types of cells in culture with better performances than commercial polymeric supports [7].

The work we present here describes the use of inorganic and MMMs zeolite membranes as scaffolds for in vitro analyses of olive oil-containing cosmetics. The basis of this approach is that zeolite membranes with their high surface area and their crystalline porous channel system permit the free passage of culture media cations, substances, and water determining higher cell densities with respect to commercial membranes. To the best of our knowledge, this is the first work addressing a reliable and useful application of pure zeolite and mixed matrix membranes as in vitro scaffolds to test novel cosmetic products.

II. MATERIAL AND METHOD

2.1 Chemicals reagents and cell lines

Potassium fluoride (ACS reagent: minimum 99%), tetrapropylammonium bromide (TPABr, purum), fumed silica (99.8%), colloidal silica (Ludox, AS-40, 40% suspension in water), aluminum nitrate nonahydrate (ACS reagent: minimum 98%) were purchased from Sigma Aldrich (USA). Polylactic polymer granules (PLA) were obtained from Cargill-Dow Inc. (USA) with the trade name of Nature Green® 2100D. This highly crystalline type mostly consisted of the L co-nomer containing less 1.47 ± 0.2% of the D co-nomer. Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), L-glutamine, penicillin/streptomycin was purchased from Eurobio (France), fetal bovine serum (FBS) was purchased from Life Technologies, (Life Technologies,
Paisley, UK), trypsin, sodium orthovanadate, dimethyl sulfoxide (DMSO), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), glutaraldehyde solution and osmium tetroxide were obtained from Sigma Aldrich (USA).

MCF-7 cells (human breast adenocarcinoma cells) and MCF-10A cells (human normal breast epithelial cells) were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). MCF-7 and MCF-10A cells were authenticated, stored according to the supplier's instructions, and used within a month after frozen aliquots resuscitations. MCF-7 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM, Eurobio, France) supplemented with 10% premium fetal bovine serum (FBS, Life Technologies, Paisley, UK) and 100 U/mL penicillin–streptomycin in a 5% CO2 environment. MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12, Eurobio, France) containing 5% horse serum, 100 U/mL penicillin, 100 mg/mL streptomycin, 100 ng/mL cholera toxin, 10 ng/mL epidermal growth factor, 0.5 mg/mL hydrocortisone, 10 mg/mL insulin, and 1% L-glutamine. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO2–95% air. After growing to 90% confluence, the cells were washed with Phosphate Buffer Solution (PBS, Eurobio, France) and replaced the culture medium by 1 mL PBS.

2.2 Synthesis procedure of zeolite crystals and Membrane Preparation

2.2.1 Zeolite crystals synthesis and Zeolite Membrane preparation

Fe-S-1 zeolite crystals were prepared by the following fluoride procedure. The molar composition used to obtain MFI zeolite crystals was: SiO2: 2.4KF: 0.01Fe2O3: 0.4(TPA)2O: 33H2O. The mixture was heated in stainless steel Teflon-lined autoclaves at 170 °C for 3 days. After reaction time, the crystals obtained were filtered, washed with bidistilled water, and dried overnight at 110 °C. As-synthesized crystals were thermally treated up to 550 °C in nitrogen and in the air to obtain porous crystals. Linde type L crystals synthesis was performed using the following gel composition: 10K2O:Al2O3:0.1Na2O:20SiO2:1030H2O. The aged gel was transferred to a Teflon-lined stainless steel autoclave. The hydrothermal synthesis was performed at 180 °C for 3 days statically. The autoclave was then cooled down to room temperature. The resulting crystals were filtered with copious amounts of deionized water, before being left to dry at 110 °C overnight. The zeolite membranes used in this work (Table 1) as inorganic scaffolds were prepared according to the patented Tavolaro’s method with a diameter equal to 13 mm [8], while MMMs preparation were reported elsewhere [7].

<table>
<thead>
<tr>
<th>Molar atomic ratio</th>
<th>Fe-S-1</th>
<th>Fe-ZSM-5</th>
<th>Linde type L</th>
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<tbody>
<tr>
<td>Si/Al</td>
<td>∞</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Si/Fe</td>
<td>50</td>
<td>50</td>
<td>∞</td>
</tr>
<tr>
<td>Si/K</td>
<td>0.4</td>
<td>0.4</td>
<td>1</td>
</tr>
<tr>
<td>Fe/Al</td>
<td>∞</td>
<td>0.4</td>
<td>∞</td>
</tr>
<tr>
<td>PZC</td>
<td>5.02</td>
<td>6.55</td>
<td>9.4</td>
</tr>
<tr>
<td>Length a (μm)</td>
<td>11.30</td>
<td>18.43</td>
<td>2.2</td>
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<tr>
<td>Length b (μm)</td>
<td>11.30</td>
<td>6.93</td>
<td>1.7</td>
</tr>
<tr>
<td>Length b (μm)</td>
<td>11.30</td>
<td>6.36</td>
<td>1.6</td>
</tr>
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2.3 Cosmetic base cream preparation

The cosmetic cream used like the source is a newly formulated O/W emulsion (base), which was found stable after evaluating for pH, electrical conductivity, centrifugation, phase separation, temperature stability tests (data not shown). It was prepared according to a commercial formulation kindly suggested by SA.TE.CA. S.r.l, a local company that produces skin care products using sulfurous hyperthermal water [9].

2.4 Characterization of Pure Zeolite Membranes and MMMs

The crystalline zeolite frameworks of the materials prepared were identified by powder X-ray diffraction (XRD) patterns on a Philips Model PW 1730/10 generator equipped with a PW 1050/70 vertical goniometer (using Cu Ka radiation). The diffractograms were measured from 5 to 45° using a step size of 0.02 and a scanning speed of 2° min⁻¹. The calcination stage to eliminate the template utilized in the syntheses was performed in a Lindberg/blue STF55346C tube furnace in a static atmosphere. The zeolite morphologies and crystal sizes were determined by a FESEM, FEI – Philips Quanta 200. FTIR-ATR
spectra were directly collected from the membrane surfaces over different points of the sampling area at the same pressure with a micrometer torque (UATR crystal Diamond/ZnSe Spectrum One System by Perkin Elmer Instruments) equipped with an attenuated total reflectance accessory (ATR). This is an infrared technique particularly useful for surface analysis of membranes. For each sample, six scans were signal-averaged at a resolution of 4 cm\(^{-1}\). The analysis was performed on the two different surfaces of each prepared membrane.

2.5 Cell adhesion and Cell Proliferation assays

MCF-10A and MCF-7 cells were cultured with a density of 1x10\(^5\) cell/mL in no treated 24-well plates on zeolite scaffolds. Cell adhesion was determined, after three hours from seeding, by cell count using a Burker’s chamber and Polarized Light Photomicroscope and Field Emission Scanning Electron Microscope analysis. The cosmetic emulsion concentration in DMSO used in treatments was equal to 8 µg/mL. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) at 24 h post-treatment. MTT solution (5 mg/mL, Sigma Aldrich, Milan, Italy) was added to a volume of 1 mL in each well and was incubated for three hours. Then, the solution was removed, and 100 µL of DMSO was added to solubilize the crystals. The wells were then read by spectrophotometer at the wavelength of 570 nm (Olympus Instruments, Japan). The results are representative of at least three independent experiments for each cell line. The percentage of viable cells was calculated according to our previous study.

2.6 Microphotography and Imaging analysis

The morphology of MCF-10A and MCF-7 cells in different culture conditions was studied by Polarized Light Photomicroscope (PLP) and Field Emission Scanning Electron Microscope (FESEM) analyses. Samples of both cell lines were prepared for FESEM by fixation in 2.5% glutaraldehyde, pH 7.4 phosphate buffer, followed by post-fixation in 1% osmium tetroxide and by progressive dehydration in ethanol. For observations and imaging analysis, we used a FESEM, FEI-Philips, Quanta 200 equipped with detector EDX and a Polarized Photomicroscope BX 41-MLED (Olympus Instruments, Japan) equipped with camera SC30 CMOS colors 3MPIXELS and Cell A Image Plus software (Olympus Instruments, Japan). Data were expressed as the mean ± standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the Bonferroni’s multiple comparison tests. The level of significance at * p-value < 0.05 was considered statistically significant.

III. RESULTS AND DISCUSSION

Here, we fabricated novel MMMs and pure zeolite membranes substrates for MCF-10A and MCF-7 growth to observe the possible toxic and/or inhibitory responses of epithelial and tumor cell activity after the administration of novel olive oil-containing cosmetics. Table 1 shows Si/Al, Si/Fe, Si/K, and Fe/Al ratios, PZCs and crystalline dimensions for as-made zeolite crystals prepared and used in this work. The powder X-ray diffraction (XRD) patterns of Fe-S-1, Fe-ZSM-5, and Linde type L zeolite crystals synthesized and used in this work to prepare both pure and hybrid membranes are shown in Figure 1 a, b and c, respectively. These figures exhibit the typical diffraction patterns of MFI and LTL type zeolites with high crystalline structures that matching well with the XRD powder patterns both simulated and reported in the literature, and indicate the successful syntheses [10], FESEM microphotograph of Fe-S-1 crystals and surface pure membrane are shown in Figure 1 (d and e). EDX analyses of the zeolite crystals used to prepare all membranes showed different chemical atom ratios and compositions (Figure 1f and 1g). In particular, it is possible to observe the presence of the peak at about 0.5 KeV, which corresponds to Fe Lα, and the peak at about 6.4 KeV, which is characteristic of Fe Kα radiations, that evidences the inclusion of iron atoms in the prepared hybrid zeolite scaffold. EDX spectrum of the crystalline as-made Linde type L sample evidences the presence of sodium and potassium atoms (Figure 1g). These images evidence the regular shape and morphology of the zeolite crystals forming the crystalline scaffold. Morphologies of Fe-containing MMM scaffolds can be observed in Figures 2 a-d that illustrate the surfaces and cross-sections of Fe-S-1 and Fe-ZSM-5 membranes. In these figures it is possible to evidence the different shape of zeolite crystals forming the scaffolds; in fact, Fe-S-1 membrane reveals the inclusion of regular spheric-like crystalline clusters, while Fe-ZSM-5 scaffold shows prismatic crystals embedded in the polymeric matrix. Both pure and hybrid zeolite membranes have been screened by Fourier transformed infrared spectroscopy (FTIR) to evidence typical absorption bands of synthetic crystalline zeolite membranes and the interactions between inorganic siloxane groups of crystals and polymeric matrix involved in prepared MMMs.
Figures 3, and 4 reveal the vibrational spectra of Linde type L, Fe-S-1, and Fe-ZSM-5 crystals. The FTIR spectra of Fe-containing crystalline zeolite membranes show the same typical absorbance shapes as the MFI zeolite structures. In fact, in these spectra all the vibrational bands attributed to lattice modes appear with two additive shoulders centered at about 960 cm$^{-1}$ and 1010 cm$^{-1}$, respectively. These two low bands were attributed in the literature to defective stretching of the silanol groups due to the polarization of the Fe-O-Si bonds caused to the presence of iron atoms in the zeolite frameworks [11]. According to the literature data, it is possible to observe that the bands, centered at about 750 cm$^{-1}$ and 550 cm$^{-1}$, shift to
lower wavenumbers in the presence of the aluminum atom inclusion in the zeolite framework and the resulting decrease in the Si/Al ratio. The FTIR spectrum of Linde type L closely agrees to the literature [12]. The infrared spectrum of pure PLA membrane (Figure 3 b) in the region of 3000-1600 cm\(^{-1}\) is characterized by adsorption bands at 2996 cm\(^{-1}\), 2947 cm\(^{-1}\) and 2877 cm\(^{-1}\) arising from the C-H stretching vibration of methyl (CH\(_3\)) groups in the side chains (\(\nu_{as}CH_3\), \(\nu_{s}CH_3\), vCH modes) as well as a strong band related to the stretching vibration of carbonyl (C=O) groups at 1749 cm\(^{-1}\) [13]. As regards the MMMs, the infrared analysis reveals the formation of homogeneous zeolite-containing films and provides the typical bands both zeolite crystals and PLA with new modified bands, which evidence their mutual interactions. In fact, all spectra of hybrid membranes show a very similar spectroscopic behavior with the presence of both pure PLA and zeolite strong absorption bands modified. Figure 4A shows the infrared spectra of Fe-S-1 40% and Fe-ZSM-5 40% MMMs, while Figure 4B and 4C reveals the comparisons in two vibrational ranges. Infrared analyses of Fe-S-1 and Fe-ZSM-5 crystalline MMMs reveal the characteristic spectra between 1500 cm\(^{-1}\) and 400 cm\(^{-1}\) with bands of absorption centered at 1215 cm\(^{-1}\), 1087 cm\(^{-1}\), 800 cm\(^{-1}\) and 450 cm\(^{-1}\), corresponding to the Si-O-Si asymmetric stretching, symmetric stretching, and symmetric bending, respectively [14]. Figure 4 reveals the enlargements of the region comprises between 3000 and 2800 cm\(^{-1}\) (B) and from 580 up to 400 cm\(^{-1}\) (C) and evidences the shift of the vibrational bands for Fe-ZSM-5 40% with respect to Fe-S-1 40% MMMs. In particular, the intense band centered at 550 cm\(^{-1}\) is characteristic of the pentasil zeolites and it is assigned to the vibration of double 5-rings in MFI lattice; no such a band appears in the spectrum of amorphous silica material then it evidences the crystallization of structures prepared [15]. All zeolite membranes fabricated and characterized were used for cell cultures. As we have already pointed out in previous works, the synthetic zeolitic scaffolds are non-cytotoxic and stable in the cell culture medium promoting a better adhesion with respect to the polymer scaffolds. Here too, our experiments have confirmed that cells react selectively with a specific zeolitic structure. In particular, MCF-10A cells preferentially adhere and grow on the pure zeolitic membrane of Linde type L (results not shown). Figure 5 shows the histograms related to the analyses of MCF-10A (A) and MCF-7 (B) cells grown on scaffolds prepared in this work in the absence and after 24 h of treatment with the cosmetic emulsion. Data obtained reveal that they favor better viability than the polystyrene (PS) control scaffold. Moreover, inorganic zeolite membranes show better viability with respect to MMMs for both cell lines. In particular, cancer cells grow better than normal cells, but after the cosmetic administration, the two cell lines show a different behavior. In fact, while MCF-10A undergoes increased cell viability, MCF-7 shows a decrease mainly on membranes. This behavior can be due to the effect of the cosmetic on the decreasing of oxidative stress in non-tumor epithelial cells and on the improving the physiological conditions, while the decrease in vitality in carcinogenic cells after administration may be caused by an increase in cosmetic-induced apoptosis.

![Figure 5](image-url)

**FIGURE 5.** Cell viability determined by MTT test on MCF-10A (A) and MCF-7 (B) treated for 24 h with cosmetic (8 
\(\mu\)g/mL). Columns are the mean of three independent experiments each in triplicate; bars ± S.D. (n = 4); *\(p < 0.05\)
untreated cells vs PS; ●\(p < 0.05\) treated cells vs PS.
IV. CONCLUSION

The results reported in this article highlight the peculiarities of zeolitic scaffolds prepared as excellent cellular supports for the analysis and control of the cytotoxicological characteristics of cosmetics. A comparison between zeolite crystals-containing hybrid and pure membranes with the same framework, crystal pore and dimensions reveals that the density of cells adhered and grown on supports was always greater for inorganic scaffolds with respect to the polymeric surfaces suggesting that siloxane groups act as binding sites for the cellular membrane.

Our experimental data demonstrate that all cell adhesions are membrane-specific and, in particular, that MCF-10A cells preferentially interact with pure zeolitic membranes. Furthermore, the viability data obtained reveal that pure inorganic supports promote greater cell growth both in respect to the polystyrene control scaffold (PS) and to the MMMs membranes, for two cell lines. After administration of the solid olive oil-containing cosmetic, the normal cells show a better viability, but the tumor cells undergo an evident decrease.

In conclusion, it is evident that due to their chemical-physical peculiarities, simplicity of preparation, biocompatibility and chemical stability in both physiological and conditioned culture media, the prepared zeolitic scaffolds are excellent supports to easily determine the anticancer characteristics of novel cosmetics of natural origin.

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REFERENCES