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# Anticancer activity modulation of an innovative solid formulation of extra virgin olive oil by cultured zeolite scaffolds

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ABSTRACT

This paper deals with the design and manufacture of pure and hybrid synthetic (Mixed Matrix Membranes, MMMs) zeolite scaffolds (containing various amount of zeolite crystals dispersed in a polymeric matrix) to obtain new biomaterials. These scaffolds can potentially be used in the field of translational medicine to obtain innovative results to address tumorigenesis mechanisms with the promotion of an effort to deal with technical methods and information. Since olive oil has beneficial effects in healthy human cells and slows down and/or inhibits cell growth, the aim of this work was to monitor the protective and beneficial antitumor effects of olive oil in a new solid formulation (Spread Bio-Oil) on cancer cell cultured on zeolite scaffolds. In order to investigate the cytotoxicity of the new bio-oil spread and to test antiproliferative activity on the cancer cells we used two phenotypically different human breast cancer cell lines (MCF-7 and MDA-MB-231) seeded on various morphologies of zeolite membranes. We report the fabrication and characterization of pure and hybrid (MMMs) zeolite membranes and evaluated the intensively cell adhesion, spreading and cell growth by adhesion test, MTT, optical microscopy analyses and Scanning Electronic Microscopy (SEM) microphotography analyses. Our results demonstrate that both cell lines adhered and grow on all zeolite surfaces and that both show better viability after Spread Bio-Oil treatments. All cell adhesions are a specific membrane-type and, in particular, MCF-7 cells interact and adhere preferentially on pure zeolite membranes. Cancer cells seem to recognize and prefer the characteristics of the supports according to the following trend: Co-ZSM-5 > Co-S-1 > 13X. Moreover, Co-ZSM-5 zeolite membranes were the best scaffolds and MDA-MB-231 cells after administration of Spread Bio-Oil showed less viability with respect to MCF-7 responding better to all concentrations of the innovative food. Our data indicate that Spread Bio-Oil decreases at very low concentration values (5, 10, 25, 50, 100, 200 and 300 μg/mL) cell proliferation in a dose- and time-dependent manner. The work confirms both the superiority of pure zeolite scaffolds for cultures of human normal and cancer cells and Spread Bio-Oil as an innovative food preserving all the beneficial and healthy properties of the extra virgin olive oil from which it derives.

## 1. Introduction

Today, cancer is a major health problem worldwide. However, countries in the Mediterranean area have a lower percentage of patients and above all of deaths from this pathology than in the rest of Europe and the United States (Keys et al., 2017; Youlden et al., 2012). In particular, breast cancer is the most frequent malignancy in women in North America, with most of the deaths recorded due to metastases, rather than to localized tumor. The large geographical variation in breast cancer incidence, apart from possible genetic factors, is related to different lifestyle factors, including diet. Moreover, breast cancer, similar to most cancers, is heterogeneous in its pathology, natural history and response to treatment. Breast cancer differs, for example, with respect to estrogen (ER) and progesterone receptor (PR) status; hormone receptor-positive cancers have a better differentiated morphological appearance and stronger clinical response to hormonal treatment (Althuis et al., 2004). There is evidence to suggest that risk factors (Colditz et al., 2004), including food items (Cho et al., 2007; Stripp et al., 2003) (Olsen et al., 2003) and dietary patterns (Agurs-Collins et al., 2009; Fung et al., 2005; Velie et al., 2005) differ in their association with tumor receptor status.

The traditional Mediterranean diet discovered by Ancel and

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Margaret Key (Dixon and Blackburn, 2015) and recognized by UNESCO as an intangible cultural heritage of humanity, is characterized by high consumption of foods of plant origin, relatively low consumption of red meat, and high consumption of olives and their products. Olive oil is the fat of choice in the Mediterranean area. The most representative fraction of olive oil is polyphenolic compounds (Carrasco-Pancorbo et al., 2005). The beneficial and protective effects of olive oil against human breast cancer exerted by the phenols, including oleuropein, oleocanthal, hydroxytyrosol, tyrosol, secoiridoids, and lignans (Owen et al., 2000) were supported by multiple in vitro as well as in vivo studies. The first, in vitro evidence of the antiproliferative effects of polyphenols in three breast-derived cancer cell lines was obtained by Damianaki (Damianaki et al., 2000). His results evidenced that polyphenols, at the picomolar or the nanomolar range, decrease cell proliferation in a dose- and time-dependent manner and indicated that low concentrations of polyphenols, and consecutively, consumption of wine, or other polyphenol-rich foods and beverages, could have a beneficial antiproliferative effect on breast cancer cell growth. In addition, Nifli (Nifli et al., 2018) characterized four polyphenols (major constituents of wine) in the hormone-sensitive human cancer T47D cell line, showing the potential antiproliferative and cell cycle arrest activity of polyphenols. Goulas (Goulas et al., 2009) investigated the antioxidant potency and antiproliferative activity against cancer and endothelial cells of olive leaves extracts in breast cancer cells. Oleuropein, phenols and flavonoids (dominant compound of the extracts) demonstrated strong antioxidant potency and inhibited cancer and endothelial cell proliferation at low micromolar concentrations. Thus, obtaining a solid compound (Spread Bio-Oil) from extra virgin olive oil able to retain all the beneficial and healthy properties of the oil from which it derives, makes this alimentary product very interesting. This hard fat phase can be used as margarine, a shortening, or butter replacer for bakery production, and/or as a ready-to-eat fat spread.

Since it is, at the same time, more easily handled, transportable and storable, and promotes a more widespread use of the most beneficial product of natural origin and constitutes an innovative food. Spread Bio-Oil has been used in the experiments the subject of this paper. Rheologically, it is a structured water-in-oil emulsion based on an oil phase containing extra-virgin olive oil.

Based on these premises, we aimed to investigate whether and to what extent, Spread Bio-Oil influences human cell neoplastic parameters.

Since there are a number of studies that show the beneficial health effects of extra virgin olive oil and it has received much attention because several epidemiological studies have shown that its consumption is associated with a reduction of breast cancer risk (Vecchia et al., 1995), (Psaltopoulou et al., 2011), we have focused our attention testing the in vitro activities of solid oil using two breast cancer cell lines with different malignancy.

Classification and staging systems are important in oncology to predict clinical behavior and determine prognosis (Kenny et al., 2007; Kriegmair et al., 2018). In addition, they contribute to the selection of optimal treatment strategies. Much clinical and translational research over the past 20 years has been directed at establishing or refining prognostic and predictive factors for breast cancer. Initially, tumor related factors such as size, grade, morphology, lymph node involvement, and hormone receptor status were considered in the determination of prognosis. Patient characteristics, such as age, menopausal status and performance status, also contributed to these estimates. Some factors such as estrogen receptor (ER) and progesterone receptor (PR) status were shown to be better predictive factors than prognostic factors. Patients with tumors that express hormone receptors have a significant increase in survival free from disease and survival overall compared to patients with receptors negative hormones, although this figure is often related to the benefit obtained in these patients thanks to endocrine treatment. Generally, the hormone receptor positivity is associated with other characteristics of lesser aggression, such as a high degree of differentiation and low proliferative index (Pusztai et al., 2006). The expression of human epidermal growth factor receptor 2 (HER2), in addition to its now undisputed prognostic meaning, has an important role also as a factor in sensitivity to endocrine therapy and to chemotherapy (Guiu et al., 2013). The new molecular markers and the possibility of studying the gene profile of the single tumor open the way to an increasingly precise selection of patients actually at risk of disease recovery, as well as a more precise use of therapeutic resources thanks to the identification of markers able to predict the sensitivity or resistance to different drugs.

This study utilizes estrogen receptor (ER)-positive, poorly invasive, and low metastasizing human breast carcinoma cell line MCF-7 and triple negative, highly invasive, and metastatic human breast carcinoma cell line MDA-MB-231 (Ford et al., 2011).

The cells were seeded and adhered on zeolite membranes to analyze both the cytotoxicity of the scaffolds and of the innovative olive food and how it influences neoplastic growth and differentiation under different conditions.

Zeolites are known to be stable both in wet and dry conditions, do not favor the development of microorganisms, are compatible with biochemical analysis and, owing to their high biocompatibility, today they are considered new biomaterials. They are inorganic materials with, a highly ordered structure and can be synthesized with different crystalline size. As inorganic support materials to immobilize biological species or to culture cells, zeolites have interesting characteristics, such as mechanical and chemical resistance as well as a large surface area (Tavolaro and Drioli, 1999). The innovative exploitation of the intima structure of non-functionalized zeolite scaffolds in cell cultures is the goal of this paper for the in vitro analysis of food, drugs, cosmetics, nutraceuticals, etc. These crystalline, inorganic and chemically stable biomaterials in conditioned culture media do not interfere with the cell activity. This enables study of the carcinogenicity of a molecular species, pure or in mixture. Substances added as aggregates and additives can also be tested to analyze their healthiness and to control the maintenance of the quality of the original food product (such as the chemical activity of the polyphenols contained in the novel spread olive oil formulation), as added stabilizer species.

## 2. Materials and methods

### 2.1. Reagents

Zeolite materials were prepared using tetraethyl orthosilicate (JANSSEN), tetrabutylammonium bromide, aluminum nitrate nonahydrate (ALDRICH), Silica fumed (SIGMA), potassium fluoride (ACS reagent: minimum 99%), tetrapropylammonium bromide (JANSSEN) and cobalt sulfate (BAKER). Sodium hydroxide and sodium fluoride were purchased from Carlo Erba Reagents (Italy). The crystals obtained were used to synthesize new zeolite pure and hybrid (MMMs) membranes: Co-S-1, Co-ZSM-5, and 13X.

Polylactic polymer granules (PLA) were obtained from Cargill-Dow Inc. (USA) with the trade name of Nature Green® 2100D. Phosphate buffered saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12), L-glutamine, and penicillin/streptomycin were 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-diphenyltatrazolium bromide), glutaraldehyde solution and osmium tetroxide were obtained from Sigma Aldrich (USA).

### 2.2. Spread Bio-Oil

Spread Bio-Oil is a water-in-oil emulsion based on an oil phase primarily containing extra-virgin olive oil (Gabro, Italy) for more than 60%w/w, cocoa butter (Icam, Italy), and Myverol (50% of glyceryl

monostearate and 50% glyceryl monopalmitate, Kerry Group, Ireland). Distilled water is the aqueous phase of the final emulsion. The oil phase is produced by heating olive oil and mixing cocoa butter and monoglycerides; finally, emulsification is carried out at low temperatures with water. Further details can be found in the patent WO 2013111058 A1 by De Cindio et al. (Petramale et al., 2013).

Spread Bio-Oil was dissolved in 99% ethanol, stored in the dark as stock solution ($1 \times 10^{-2}$ g/mL) and diluted with growth medium before the treatments. Control cells were cultured in medium containing the same concentration of ethanol (v/v) as the experimental cultures with Spread Bio-Oil. The ethanol solution had no noticeable influence on the proliferation of the cancer cells.

### 2.3. Breast cancer cell lines

Human breast cancer MCF-7 and MDA-MB-231 cells and human mammary epithelial MCF-10A cells, used as comparison, were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). Both cell lines were authenticated, stored according to the supplier's instructions and cultured in Dulbecco's Modified Eagle's Medium (DMEM and DMEM/F12, Eurobio, France) containing antibiotics (100 IU/ml) penicillin and (100 mg/ml) streptomycin or gentamicin (50 μg/ml) (Eurobio, France), supplemented with 10% FBS, and 10 mM HEPES serum (FBS, Life Technologies, Paisley, UK). All the cells were maintained in a humidified 5% $CO_2$ incubator at 37 °C.

### 2.4. Preparation and characterization of zeolite scaffolds

The zeolite crystals were prepared by hydrothermal syntheses, calcined and then used for the preparation of zeolite membranes. In particular, zeolite crystals were used both as crystalline seeds for the preparation of pure membranes and active solid components in the polymeric phase of hybrid membranes (Mixed Matrix Membranes, MMMs), respectively.

Cobalt-containing zeolite crystals were prepared using fluoride gels, and a traditional high-pH gel was performed to synthesize 13X zeolite. The molar composition used to obtain Co-S-1 zeolite crystals was: $SiO_2$: 24 KF: 0.2$CoSO_4$: 2$(TPA)_2O$: 33$H_2O$, while the 0.65$(TPA)_2O$ and 0.2Al$(NO_3)_3$ relative molar content were used to synthesize Co-ZSM-5 zeolite crystals.

The precursor mixture was heated in stainless steel Teflon-lined autoclaves at 170 °C for the time necessary to obtain complete crystallization (3 days for Co-containing framework) without mechanical stirring. After reaction time, the crystals were filtered, washed with bi-distilled water, and dried overnight at 110 °C. As-synthesized crystals were thermally treated (calcined) in nitrogen to obtain microporous crystals. The Si/Al and Si/Co ratios in Co-S-1, Co-ZSM-5 crystals were equal to ∞, 9.70, 166 and 26, respectively. The Si/Al ratio in 13X zeolite crystals was 1.55.

The preparation of pure zeolite membranes with a diameter of 13 mm was carried out according to Tavolaro's method (Tavolaro et al., 2011).

Hybrid scaffolds were fabricated by the solvent evaporation method at 40 °C for 48 h (Tavolaro et al., 2016a). Crystalline zeolites and polylactic acid particles (PLA) (40% wt./wt.) were dispersed in chloroform then the mixture was magnetically stirred for 12 h at room temperature. The dense membranes, which were detached in distilled water and dried, were transparent with a thickness of approximately 25 ± 4 μm. The measurement of membrane thickness was performed using a digital micrometer (Mahr 40E, Germany) with the accuracy of ± 4 μm and confirmed by SEM microphotographs.

All samples were characterized by Fourier Transform Infrared Attenuated Total reflection (FTIR ATR), X-Ray Diffractometry (XRD), scanning electron microscopy (SEM) and Energy Dispersive X-ray (EDX) analyses.

### 2.5. Cell adhesion

MCF-7 and MDA-MB-231 cells were cultured with a density of $1 \times 10^5$ cells/mL in non-treated 24-well plates on zeolite scaffolds. Cell adhesion was determined, after 3 h from seeding, by cell count using a Burker's chamber and Polarized Light Photomicroscope and Field Emission Scanning Electron Microscope analysis.

### 2.6. Cell viability assay

MCF-7 and MDA-MB-231 cells were seeded in non-treated 24-well plates at a concentration of $1 \times 10^5$ cells/well and maintained in serum containing medium. In order to verify the tumor-activity and safeguard due to the use of innovative food, 5, 10, 25, 50, 100, 200 and 300 μg/mL of Spread Bio-Oil were administrated in both the cell lines grown on different zeolite scaffolds just here synthesized. Cell viability was determined by two different methods: Acridine Orange (AO) test and MTT assay. AO (Molecular Probes, Eugene, cat. no. A1301) stock solution was prepared in water (1 mg/mL) and stored at 4 °C. Aliquots of the stock solutions of the dyes were added directly to the culture media. Prior to imaging, cells were incubated with AO solution for 5 min. AO method (2.6 mM in culture medium) as reported in the literature (Ichimura, 1975) is based on the metachromatic capacity to emit fluorescence. Red fluorescence is observed when it penetrates into the acid compartments of the cell highlighting cell damage, while green fluorescence is revealed when it is free in the cytoplasm highlighting the integrity of cell membranes. The orthochromatic emission is at 540 nm and the metachromatic emission at 600 nm. In these experiments, we used an Olympus LX 50 inverted fluorescence microscope. The photomicrographs were acquired by a camera connected to the computer using the Olympus Cell-A imaging software. The images are analyzed by the NIH Image J1.61 software.

The second method, MTT assay, is based on the reduction of tetrazolium salt to formazan crystals by living cells. MTT solution (5 mg/mL, Sigma Aldrich, Milan, Italy) was added at a volume of 1 mL in each well and was incubated for 3 h. 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 following formula was used to determine the percentage of viable cells:

Percentage of cell viability = [OD Sample/OD Control] x 100%

### 2.7. Microphotography and imaging analysis

MCF-7 and MDA-MB-231 morphological characteristics were observed and analyzed, for all treatment conditions, 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 an FESEM, FEI Philips, Quanta 200 equipped with EDX detector and a Polarized Photomicroscope BX 41-MLED (Olympus Instruments, Japan) equipped with SC30 CMOS colors 3MPIXELS camera and Cell A Image Plus software.

### 2.8. Statistical analysis

Data was expressed as mean ± standard deviation (S.D.) of at least three independent experiments. Statistical analysis was performed using one-way analysis of variance (ANOVA). The level of significance at *p-value < 0.05 was considered statistically significant.

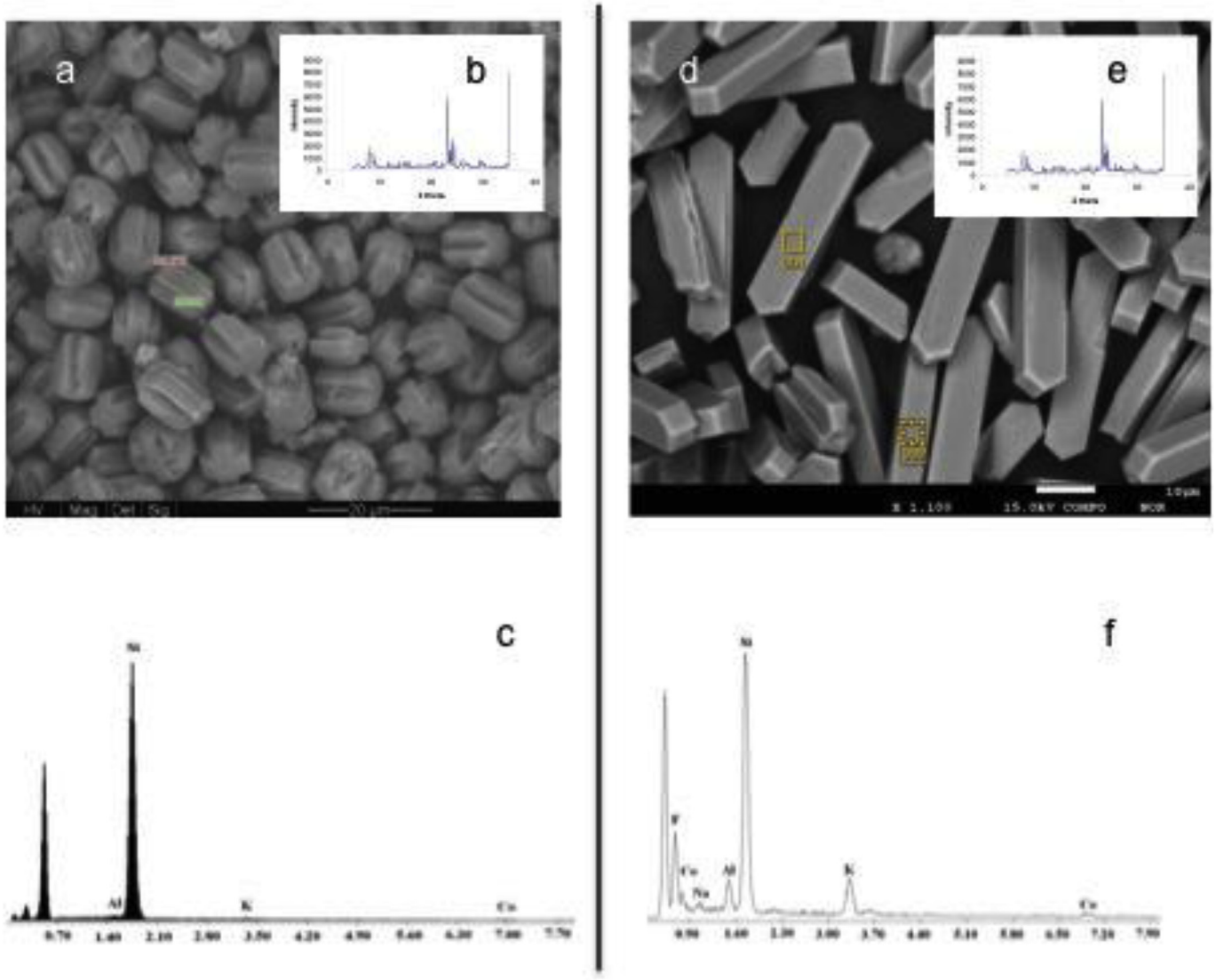



**Fig. 1.** Co-containing zeolite crystal synthesized: a) Co-S-1 zeolite crystals FESEM microphotograph, b) XRD analysis and c) EDX pattern; d) Co-ZSM-5 zeolite crystals FESEM microphotograph, e) XRD analysis and f) EDX pattern.

## 3. Results

In order to verify a possible correlation between protective and antineoplastic effects of olive solid on breast cancer cells, we screened its effects on the growth and morpho-physiological development of human breast cancer cells. For screening the antitumor effect of Spread Bio-Oil, different zeolite scaffolds and increasing doses of the food to treat human breast cancer cells were used. Novel MMMs and pure zeolite membranes supports for both cell lines were fabricated to observe the possible toxic and/or inhibitory responses after the administration of solid olive oil. Meanwhile, the effect on chemical composition, crystalline size and morphology, preparation method and zeolite framework on interactions with cells were studied. The structure and texture of zeolite materials were identified by X-ray diffraction (XRD), scanning electron microscopy (FESEM), and by Fourier transform infrared attenuated total reflectance (FTIR-ATR) analysis.

Fig. 1 shows the chemical physical characterization of Co-containing zeolite crystals synthesized and used in this work to prepare both pure and hybrid membranes. FESEM microphotograph of Co-S-1, the relative powder X-ray diffraction (XRD) pattern and EDX analysis are shown in Fig. 1a, b and c, respectively. Fig. 1d, e and f reveal the same analyses of Co-ZSM-5 crystals. These figures highlight the high crystallinity and homogeneity of the synthesized crystals, in addition to the typical diffraction patterns of MFI type framework.

Comparison of Fig. 1c and f highlights the difference in Al, Na and K counter-ions. In fact, Co-S-1 crystalline structure results similar to Silicalite-1, while Co-ZSM-5 has a chemical composition in accordance with a ZSM-5 framework. Infrared analysis of MMMs also supports the difference between the two types of synthesized crystals. In fact, in the characteristic range of the vibrations of the framework, the red arrows highlight the different absorbance bands (Fig. 2).

The morpho-structural characteristics and the related EDX analysis of the Co-S-1 hybrid membrane are revealed in Fig. 3a and b, respectively.

Fig. 4 shows the photomicrographs relating to the surface of pure cobalt-containing membranes. It shows that the surfaces, which will be exposed to cell growth, are homogeneous and crystalline. As far as morphology is concerned, it is very different according to the absence (Fig. 4a) or presence (Fig. 4b) of aluminum in the precursor mixture: the Co-S-1 membrane surface consists of crystals with a length of ca. 15 μm (Fig. 4c), but the presence of aluminum favors crystalline intergrowth forming geminates with an average diameter of ca. 60 μm (Fig. 4d). Moreover, it shows 13X membrane surface (Fig. 4e and f) and its characterization by EDX analyses (Fig. 4g), in which the presence of

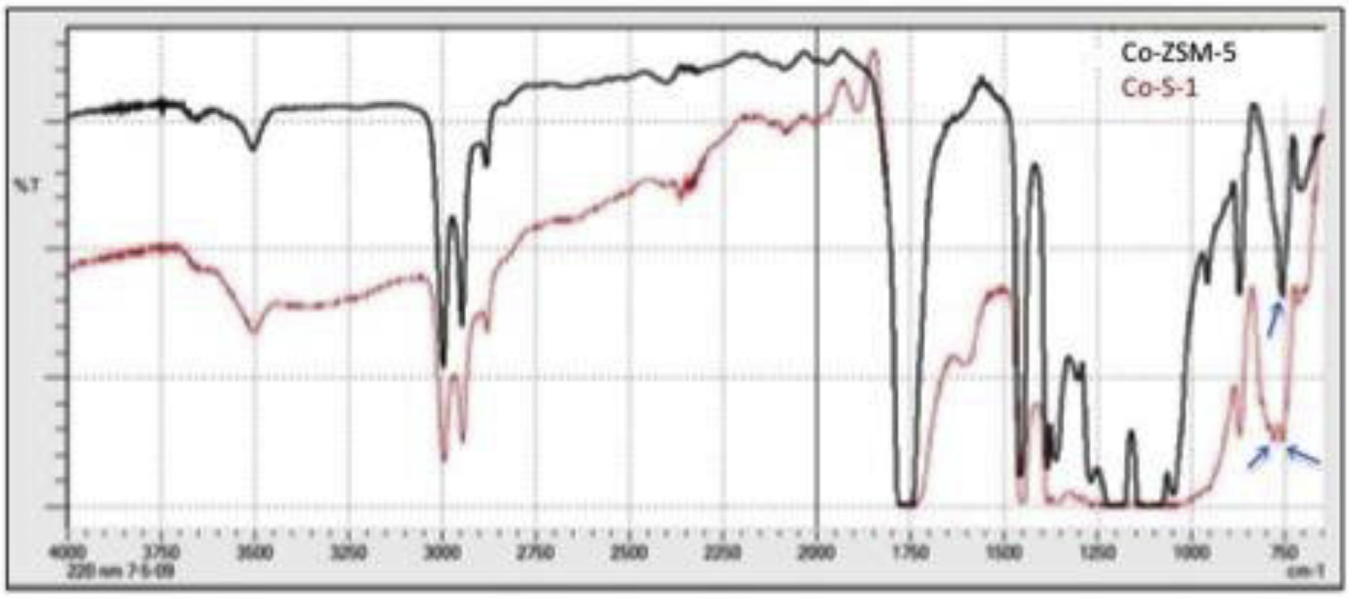



**Fig. 2.** Comparison between Co-S-1 and Co-ZSM-5 FTIR spectrum of zeolite MMMs synthesized. (MMMs = hybrid Mixed Matrix Membranes 40% wt./wt.)

aluminum and silicon is evident.

All zeolite membranes prepared and characterized were used for cell adhesion and growth. Fig. 5 shows FESEM microphotographs of MCF-7 and MDA-MB-231, adhered on Co-ZSM-5 zeolite scaffold, respectively.

The cell adhesion is a specific membrane-type and, in particular, MCF-7 cells preferentially interact and adhere on pure zeolite membranes. It indicates that the chemical-physical characteristics of the zeolite crystals, which constitute the membranes, strongly affect the interaction with both cell lines. It is important to stress that this effect is most evident for pure membranes in which the exposed surface is exclusively inorganic. Instead, in hybrid membranes, the zeolite physical-chemical peculiarities are mitigated as the crystals are embedded in the polymer matrix. Fig. 6 highlights that the various membranes have different performances in cell adhesion and that both composition (polymeric presence and alumina component) and crystalline *habitus* (shape and pore size) have a different influence on their capacity to interact with cancer cells. The experimental data obtained established that the cancer cells recognize and prefer the characteristics of the supports according to the following scheme:

Co-ZSM-5 > Co-S-1 > 13X.

After seeding in cell culture medium for 4 h, the cancer cells adhere on the zeolite membranes.

The FESEM micrographs of the MCF-7 human breast cancer cells seeded on the Co-S-1 scaffold are displayed in Fig. 7. After incubation in cell culture medium for 4 h, the cells adhere on the zeolite membrane and exhibit a round morphology (Fig. 7a), which corresponds to the cellular phenotype. After incubation for 6 h, the cells become larger and tapered in multiple directions (Fig. 7b). After incubation in cell culture medium for 12 h, the cells enlarge and flatten. The filopodia become longer and thicker, making up large and long pseudopodia that are in close contact with the zeolite membrane. At this point in time, not all the cells flatten to the same level and some cells seem to transform slowly as shown in the inset of Fig. 7c. After incubation for 18 h, a number of secretory vesicles are observed on the surface and many pseudopodia extend from the MCF-7 cells in all directions as shown in Fig. 7d. With a further increase in the culturing time to 24 h, the cells lost their typical morphology and form interconnected irregular clusters that widen on the support (Fig. 7e). After incubation for 48 h, the cells

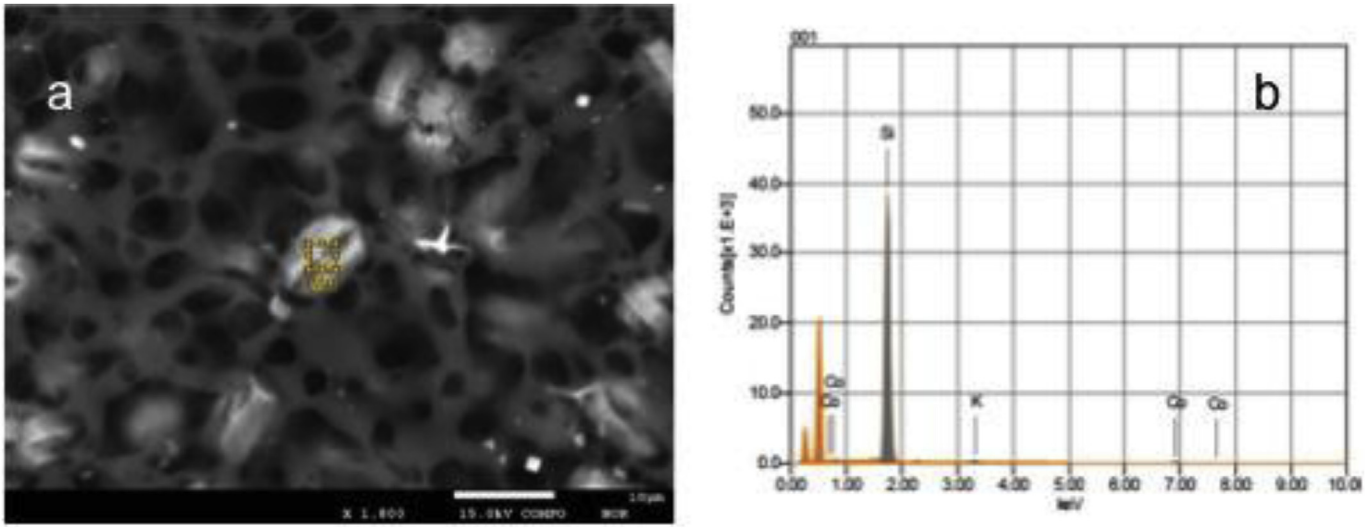



**Fig. 3.** Co-S-1 MMM prepared: a) FESEM microphotograph of the membrane surface and b) EDX analysis.

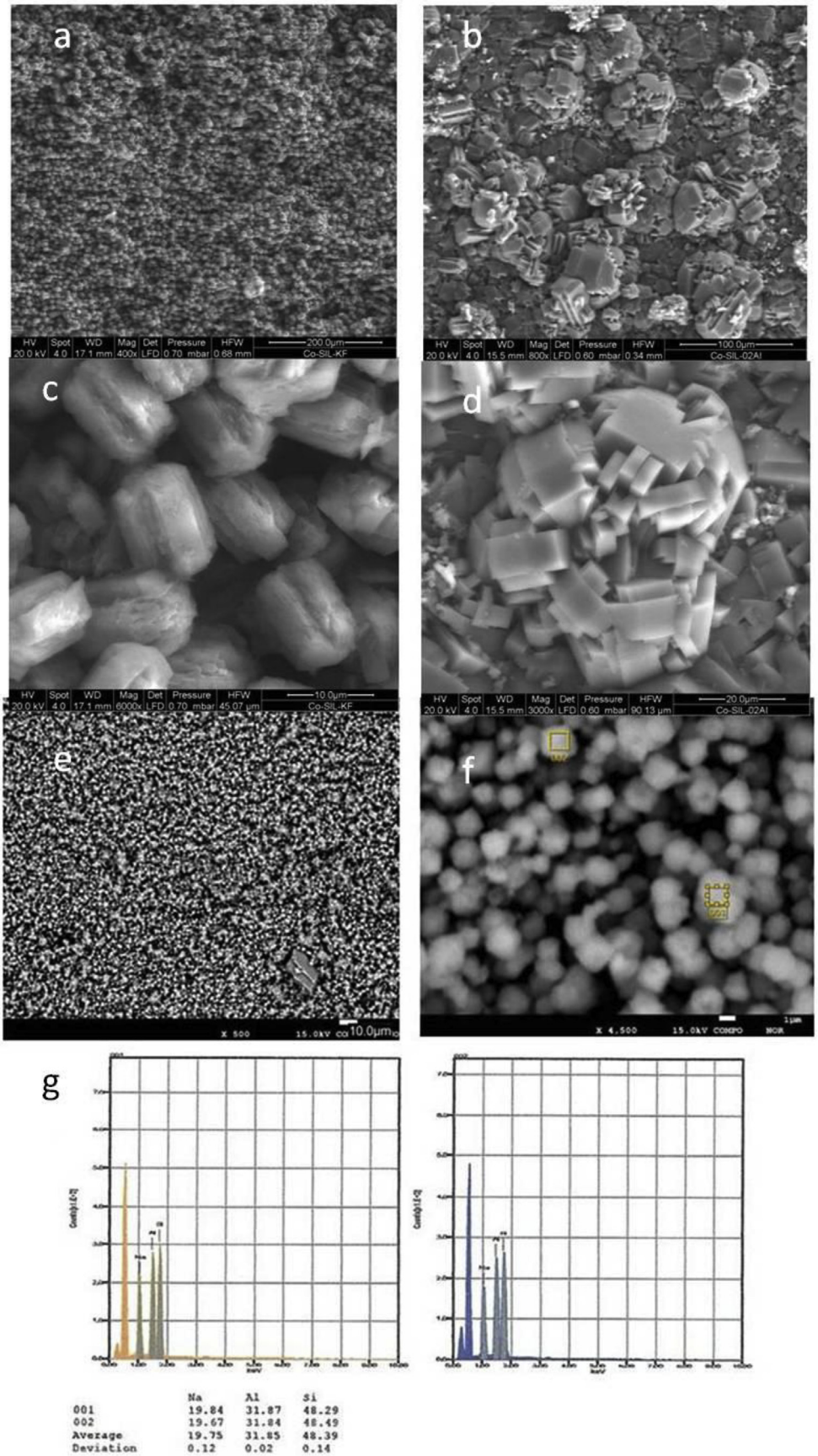



**Fig. 4.** FESEM microphotographs of pure Co-containing membrane surfaces: a) Co-S-1, b) Co-ZSM-5, c) and d) enlargements of Co-S-1 and of Co-ZSM-5, respectively; e), f) and g) 13X characterization: FESEM membrane surface, enlargement and EDX analyses, respectively.

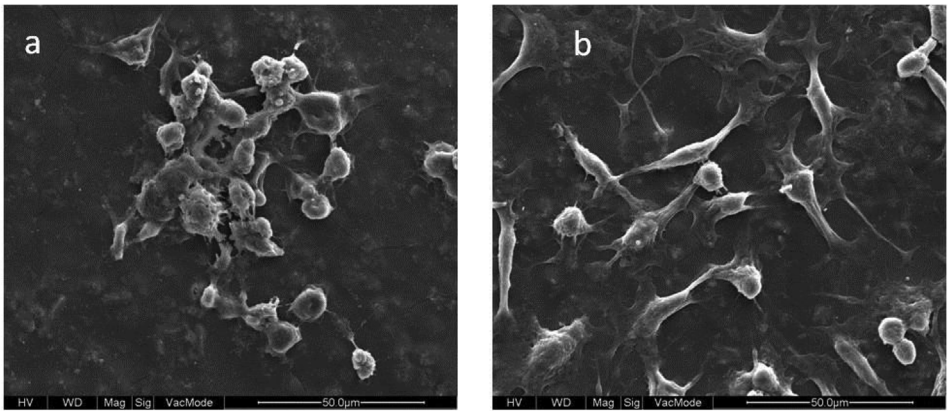
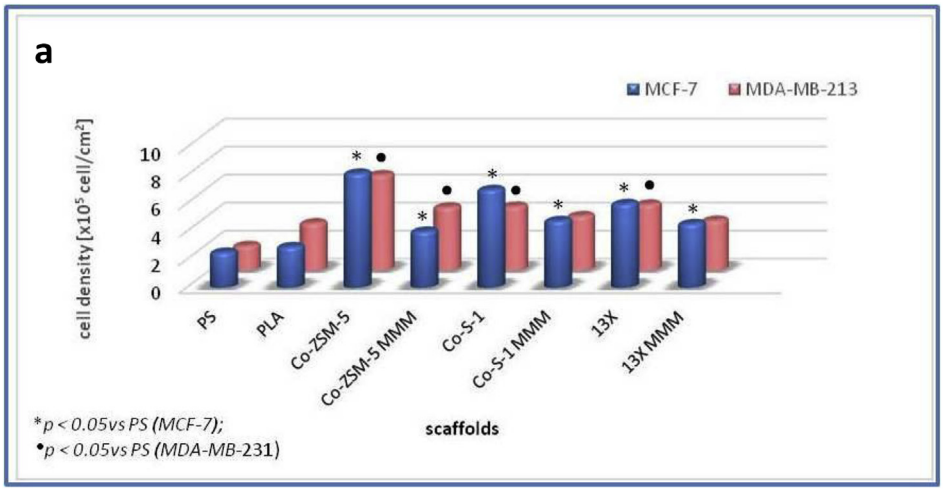



**Fig. 5.** FESEM microphotographs of MCF-7 (a) and MDA-MB-231 (b) adhered on Co-ZSM-5 zeolite scaffold.

appear to be more elongated, completely flattened and thicker (Fig. 7f). The results indicate that the MCF-7 cells can attach and adhere well to the zeolite scaffold. Cell attachment and adhesion are the first phase of cell/material interaction and the efficacy and quality of this first phase will influence the ability of the cells to proliferate and differentiate upon contact with the scaffold.

The results obtained indicate that zeolite scaffolds are noncytotoxic, easily sterilized, economic, reusable, nondegradable scaffolds for cell cultures and that they favor better adhesion than the polymeric control scaffolds.

Cell viability and apoptotic effects were performed using two tests: acridine orange and MTT. The first one was performed according to Zini



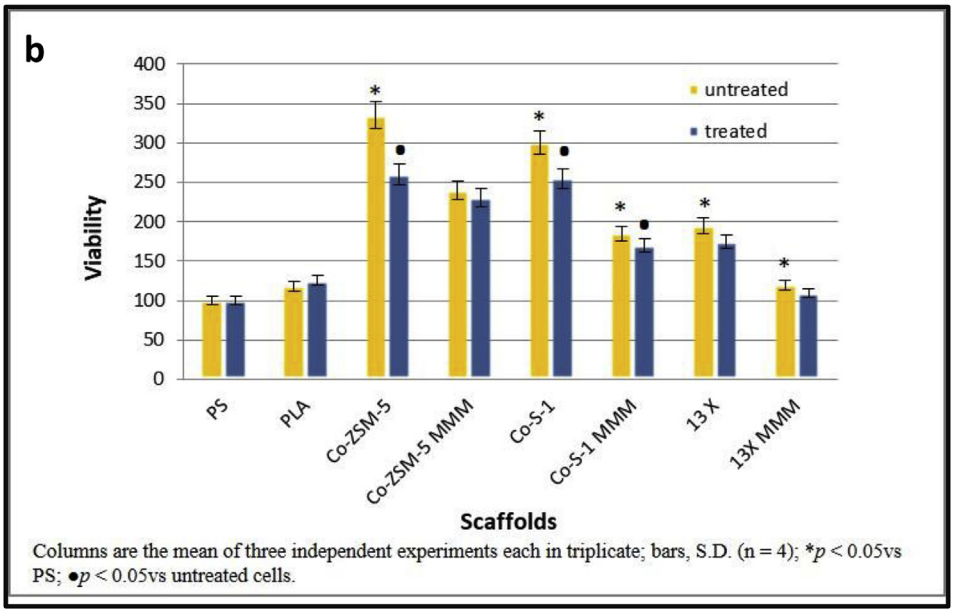




**Fig. 6.** (a) Representative histogram of adhesion of MCF-7 and MDA-MB-231 cells on synthetic zeolite membranes after 4 h from seeding. Comparison between Pure Zeolite Membranes and Mixed Matrix Membranes scaffolds. Columns are the mean of three independent experiments each in triplicate; *p < 0.05vs PS (MCF-7); •p < 0.05vs PS (MDA-MB-231). (b) Cell viability determined by MTT test on human mammary epithelial MCF-10A cells after treatment with Spread Bio-Oil 50 µg/mL for 48 h. In orange untreated and in blue treated MCF-10A. Columns are the mean of three independent experiments each in triplicate; bars, S.D. (n = 4); *p < 0.05vs PS; •p < 0.05vs untreated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

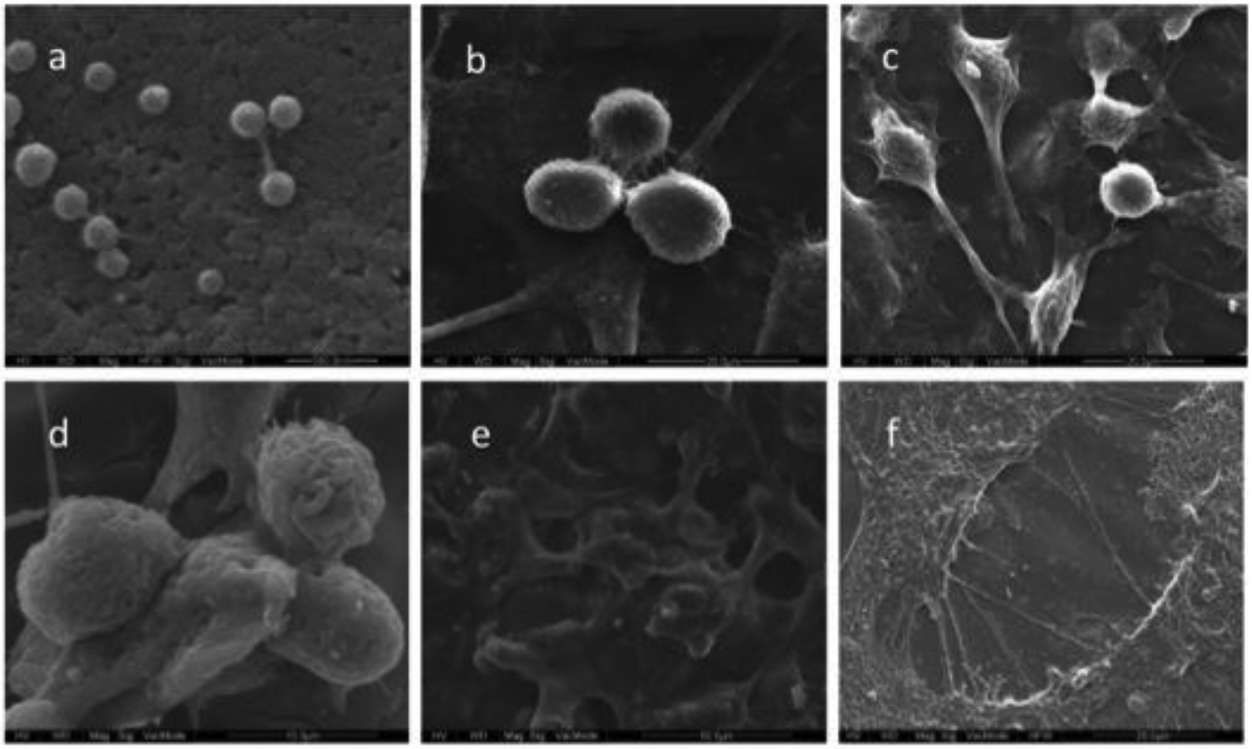



**Fig. 7.** FESEM micrographs of the MCF-7 human breast cancer cells seeded on the Co-S-1 scaffolds after various incubation time: a) 4 h, b) 6 h, c) 12 h, d) 18 h, e) 24 h and f) 48 h.

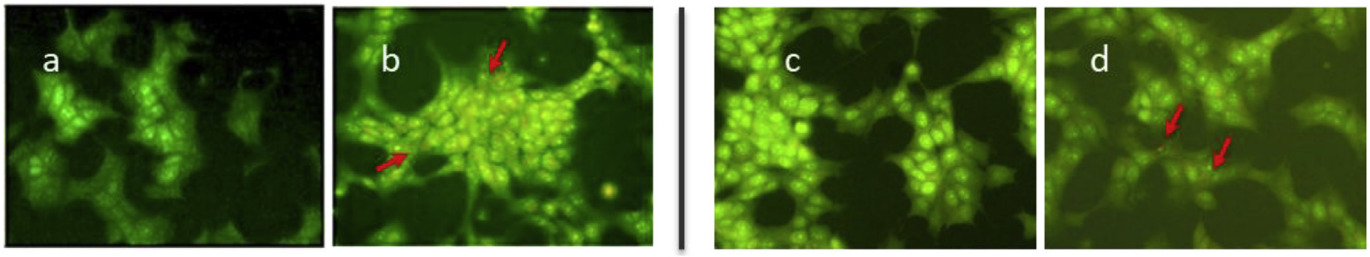



**Fig. 8.** Viability and cellular damage determined by Acridine Orange (AO) test. Fluorescence micrographs of MCF-7 (8a and 8b) and MDA-MB-231 (8c and 8d) cells on the Co-ZSM-5 pure scaffold loaded with acridine orange (2.6 mM in culture medium), demonstrating green and red luminescence of the trapped dye. Fig. 8a and c reveal human breast cancer cells without Bio-Oil Spread, while Fig. 8b and d shows cells after treatment with Bio-Oil Spread 200 μg/mL for 48 h. Red arrows highlight cellular damage. The photomicrographs were acquired by a camera connected to the computer using an Olympus Cell-A imaging software. The images are analyzed by the NIH Image J1.61 software. Red arrows highlight cellular damage. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

and Agarwal (2011) by fluorescence inverted microscopy using acridine orange/propidium iodide. This method is based on the metachromatic capacity of the dye to emit red fluorescence when it penetrates into the acid compartments of the cell highlighting (DNA and RNA) cell damage and to emit green fluorescence when it is free in the cytoplasm highlighting the integrity of cell membranes. Fig. 8 shows fluorescence images of MCF-7 (8a and 8b) and MDA-MB-231 (8c and 8d) cells on the Co-ZSM-5 pure scaffold loaded with acridine orange (2.6 mM in culture medium), with green and red luminescence of the trapped dye. Fig. 8a and c reveal human breast cancer cells without Spread Bio-Oil that appear green, while Fig. 8b and d shows cells after treatment with Spread Bio-Oil 200 μg/mL for 48 h that exhibit an obvious suffering. Red arrows highlight cellular damage.

In order to evaluate cell viability, MCF-7 and MB-MDA-231 cells, adhered and grown on zeolite scaffolds for 12 h, were treated with Spread Bio-Oil at the concentrations of 0, 5, 10, 25, 50, 100, 200 and 300 μg/mL for 12, 24, 48 and 72 h and the obtained results were summarized in the histograms in Figs. 9 and 10.

Figs. 9 and 10 clearly reveal that lower concentrations of the Bio-oil stimulate cell growth and enhance viability compared to negative vehicle-treated control cells, a pattern observed for both cell lines at all time points and through the different scaffolds. These histograms indicate a possible dose- and time-dependent activity for the Bio-Oil in treated breast cancer cell lines. In the same set of these experiments, the maximum concentration of ethanol added to culture media has been equal to 0.005 mL/mL.

The authors performed human breast cancer cell test in the presence of a well-known anti-proliferative agent, doxorubicin, using different frameworks of zeolite scaffolds. These results constitute the object of a paper published and added to the reference list (Tavolaro et al., 2016b).

## 4. Discussion

In recent decades, biodegradable polymers have been widely used as synthetic scaffolds in cancer biotherapy, tissue engineered, bioresorbable implant materials due to their biocompatibility and

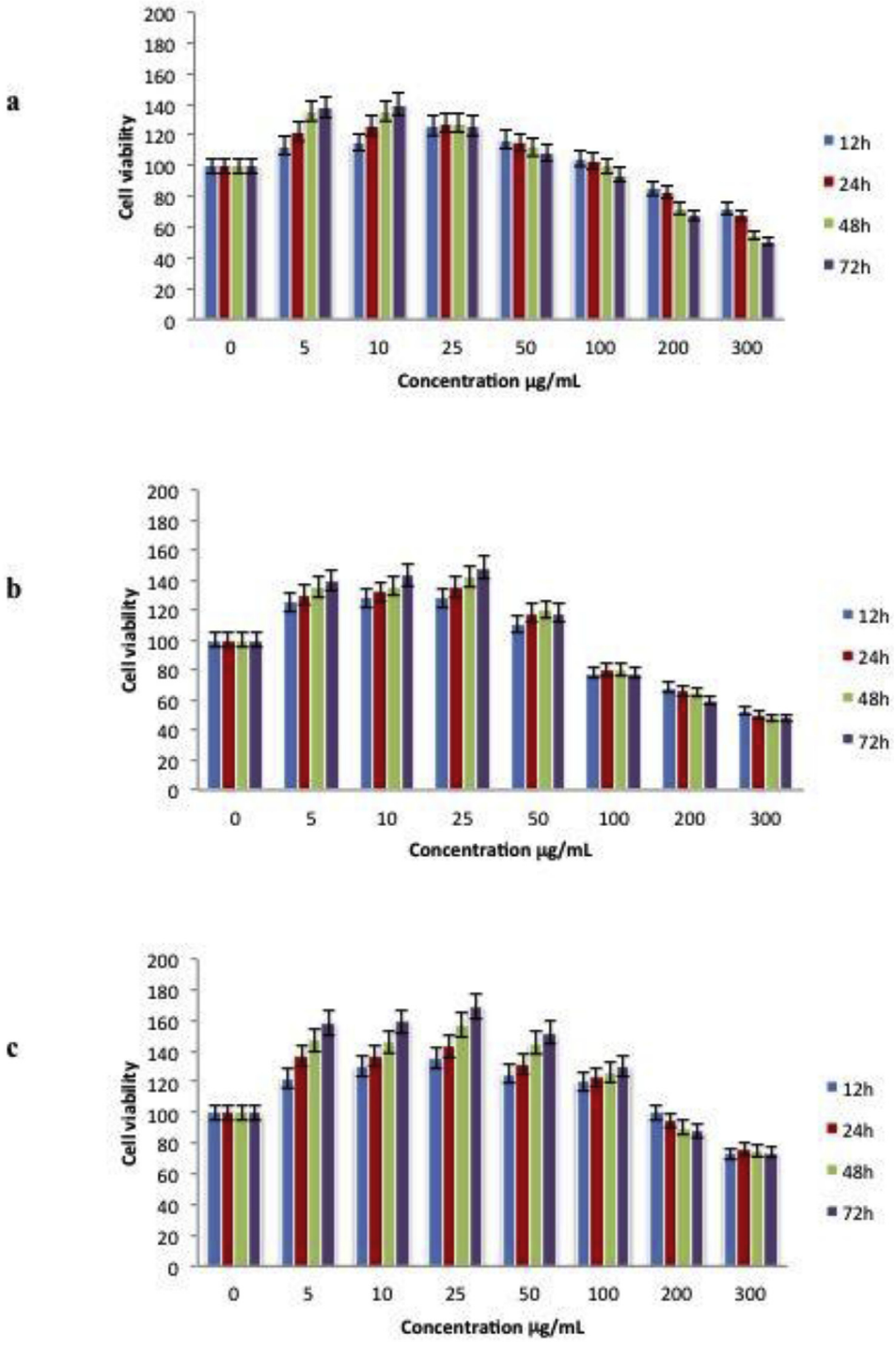



**Fig. 9.** Representative histograms of MTT assay of MCF-7 cells grown on synthetic pure zeolite scaffolds after treatment with different concentration of Spread Bio-Oil at various times: a) on Co-S-1, b) on Co-ZSM-5, and c) on 13X. Columns are the mean of three independent experiments each in triplicate; bars, S.D. (n = 4).

biodegradability. However, the rapid development of biotechnology and medical technology has required better performance from biomedical materials. New biocompatible and non-toxic materials for the production of a new generation of scaffolds with specific properties including adequate mechanical and structural support and control of cell attachment, migration, proliferation and differentiation are in great demand. Inorganic synthetic materials are preferred because they have less immunogenicity and are easily chemically changeable.

More importantly, synthetic zeolite scaffolds can be used to deliver biomolecules, such as antineoplastic drugs and growth factors, facilitating the bio-therapeutic approach.

Zeolite scaffolds synthesized with precise physico-chemical characteristics and used in experimental studies also help promote the rapid evolution of intelligent biomaterials. They can be synthesized for targeted performances (ion exchange, hydrophobic-hydrophilic modifications, acidity control) and actively contribute to the improvement of the development of effective therapies for clinical applications in various pathologies. The zeolitic membranes are membranes in which selectivity is due to the zeolitic phase. In this work, we have prepared both pure flat membranes, which are constituted exclusively of zeolitic crystals, and hybrid membranes, which are formed by a film of polylactide acid in which the zeolitic crystals are homogeneously dispersed.

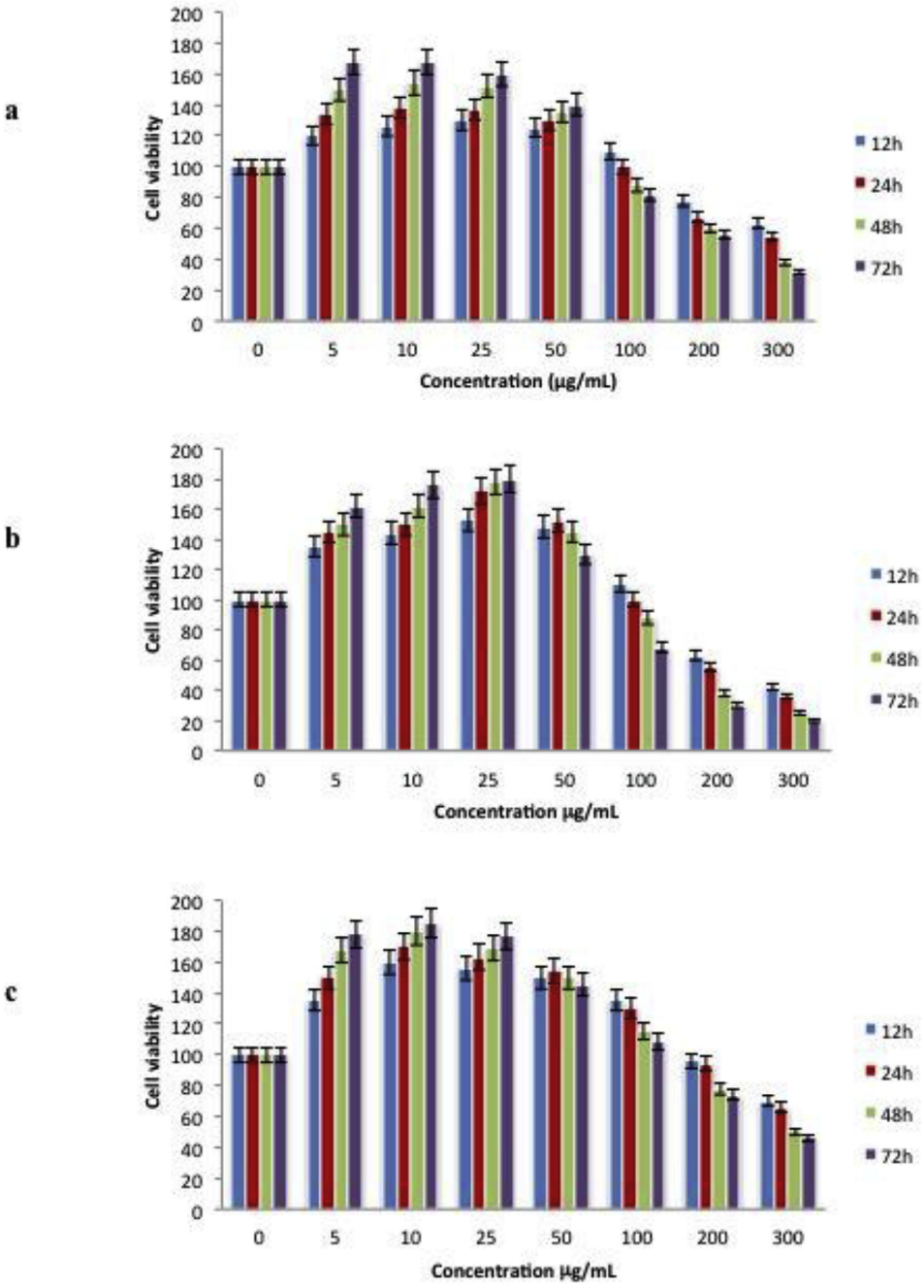



**Fig. 10.** Representative histograms of MTT assay of MDA-MB-231 cells grown on synthetic pure zeolite scaffolds after treatment with different concentration of Spread Bio-Oil at various times: a) on Co-S-1, b) on Co-ZSM-5, and c) on 13X. Columns are the mean of three independent experiments each in triplicate; bars, S.D. (n = 4).

These membranes are differentiated on the basis of the type of superficial chemical groups that can interact both with cell culture media and cells. In the case of pure membranes, the functional groups will be Si-OH, i.e. silanol, whereas in the latter case they will be those of the polymer film, which is an aliphatic polyester. These differences that determine macroscopic chemical-physical differences in membrane behavior, are altered by the influence of numerous parameters generated during the hydrothermal synthesis process of zeolite crystals (such as the type of framework, the Point of Zero Charge (PZC), the synthesis chemical medium (fluoride or basic), the acidity of Brönsted and Lewis, the hydrophobicity, the morphology, the presence of counter-cations and heteroatoms (such as Cobalt) and the chemical composition. The variation of these parameters will have a greater influence on the chemical-physical characteristics of the surfaces of pure membranes with respect to hybrid membranes and therefore on the interaction with the cells and the culture environment. Crystalline zeolites and zeolitic membranes contain crystalline microporous systems in which ions and aqueous solutions can flow across their channels influenced by the different acid properties of the synthesized crystals. For this purpose, pure membranes with variable chemical-physical characteristics were designed and created. In particular, we made membranes with different morphology of crystals, Brönsted acidity, PZC, chemical composition, isomorphous substitution with cobalt atoms, and silicon aluminum ratio. Cobalt is a transition metal that can be introduced into the framework by hydrothermal synthesis in a fluoride environment, based on its size. It is, therefore strongly bounded by covalent bonds to the

crystalline structure, which does not dissolve in the culture medium or in suspension as we have verified both for cobalt and aluminum species. The Si/Al and Si/Co ratios in Co-S-1, Co-ZSM-5 were equal to ∞, 9.70, 166 and 26, respectively. We have prepared zeolitic scaffolds as inorganic biomaterials because they are non-cytotoxic, non-degradable and insoluble in the culture environment, thermally and chemically very stable, reusable, economical materials. In the literature there are few studies on cancer cells adhered and grown on zeolite scaffolds, but with different chemical composition and frameworks (Greco et al., 2015; Tavolaro et al., 2016b), and zeolitic crystals for cancer drug delivery applications (Sagir T et al., 2016; Shin YJ and Han C-S, Lee CS,Ko S-E,Hwang SK, KO G-G, Shin JW, Yi S-K, 2010; Vilaça et al., 2013; Zhang et al., 2015).

MCF-7 and MDA-MB-231 human breast cancer cell lines were selected as model cancer cell lines because they are adherent cell lines with different characteristics. In particular, MCF-7 are estrogen receptor alpha ($ER\alpha^+$)-positive cells, HER2 negative, poorly invasive, and low metastasizing human breast carcinoma cells while MDA-MB-231 are triple negative ($ER\alpha^-$, $PR^-$, $HER2^-$) highly invasive, and metastatic human breast carcinoma cells. Estrogen receptors are primarily involved in breast cancer cell invasiveness. The invasive character of MCF-7 and MDA-MB-231 has been studied in vitro using Transwell mobility tests and Matrigel invasion tests (Justus et al., 2014). Moreover, MDA-MB-231 cells are a more aggressive and movable cellular phenotype with respect to other cells.

The influence of diet on the risk of breast cancer is of great interest as a potentially modifiable risk factor. The importance of diet in cancer etiology has been ascribed in part to the antioxidant properties of choice nutrients, influence on DNA repair, DNA mutations, metabolic detoxification, stimulation of growth factors, and potential anti-estrogenic influence of some nutrients. On the contrary, some foods have been suggested as increasing the risk of breast cancer through an increase in circulating levels of endogenous estrogen (Thomas et al., 1997).

Fat intake is related to the risk of breast cancer because it raises endogenous estrogen levels. There is evidence that diet plays a more important role in $ER^-$ breast cancer than in $ER^+$ breast cancer, but such associations cannot be detected in analyses of overall breast cancer (Fung et al., 2005; Kushi et al., 1995; Olsen et al., 2003). On the other hand, there are a number of studies on the beneficial health effects of olive oil, but no research regarding solid olive oil.

In this study, the performance of zeolite membranes was characterized with physico-chemical analyses and intensively evaluated with respect to the cell adhesion speed, attachment, spreading, and cell growth before and after treatments showing to be very fine biomaterials for cell test (Bouallagui et al., 2011; Elamin et al., 2013). The membrane that produced the best performances among all the prepared and analyzed membranes is Co-ZSM-5 pure zeolite membrane.

A comparison of zeolite crystals-containing hybrid and pure membranes with a same framework, crystal pore and dimensions reveals that the density of cells adhered and grown on supports was always greater for inorganic scaffolds compared to the polymeric surfaces suggesting that siloxane groups act as binding sites for the cellular membrane. Our experimental data evidence that all cell adhesions are of a specific membrane-type and the pure zeolite membranes are more viable compared to hybrid membranes for both cell lines. As could be expected, the MCF-7 cell line owing to its phenotypic and motility characteristics interacted better on zeolite surfaces and responded more sensitively to the administration of Spread Bio-Oil compared with the MDA-MB-231 cell line.

We have already used zeolitic scaffolds, with different characteristics, and breast cancer cells in previous studies for the administration of anticancer drugs, such as doxorubicin and bergapten to control neoplastic activity. In this work, zeolitic scaffolds were used to test a newly formulated food in order to analyze the carcinogenicity of their constituent molecular species.

This work, which is the first study of the application of zeolitic scaffolds to the quality analysis of agro-food products, highlights the great potential of these biomaterials.

We reported the healthy and protective effects of the innovative Spread Bio-Oil, which retained the beneficial properties of the olive oil from which it derives, in all experimental conditions (Leyssens et al., 2017; Simonsen et al., 2012).

## 5. Conclusions

Recently, new biotechnologies and strategies for biomaterials application in biomedicine and in health and nutrition sciences have been shifting from the synthesis of polymeric materials to the processing of similar-natural materials, which have specific physico-chemical modifiable properties. Among the most innovative biomaterials, zeolite scaffolds are prominent owing to the possibility of preparing structures and morphologies that are well suited to human physiopathological needs both in vitro and in vivo. The innovative exploitation of the intima structure of non-functionalized zeolite scaffolds in cell cultures is the goal of this paper for in vitro analysis of food, drugs, cosmetics, nutraceuticals, etc. The superiority of the pure zeolite membranes fabricated for this study was clear, confirming that zeolite scaffolds are very fine biomaterials for cell testing. Our results are interesting as they open new perspectives in the use of zeolite membranes in testing innovative alimentary products contributing to healthy feeding and, at the same time, enhancing the knowledge of useful information on neoplastic cells. Evidence from our experimental data showed that Spread Bio-Oil, added to cell culture, induced significant levels of apoptosis in both cancer cell lines. These anti-cancer properties are due to the phenolic compounds present in olive and preserved in the spreadable olive oil. This work is the first application to the cytotoxicological analysis of agro-food products on cells cultured on zeolite membranes. It demonstrates that these biomaterials constitute advanced scaffolds for future biotechnological applications in quality control systems for food products and their derivatives.

The data of the present investigation add evidence to the good choice of zeolite membranes as scaffolds for the study of cell activity. Owing to their surface structural characteristics, they may be the new biomaterials targeted to gradual adsorption and release induced by the zeolite framework. This study strongly suggests that solid oil constitutes a novel valuable phytochemical for the discovery of anticancer molecules, which could be used for the design of functional foods.

## Conflicts of interest

The authors declare that there are no conflicts of interest.

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