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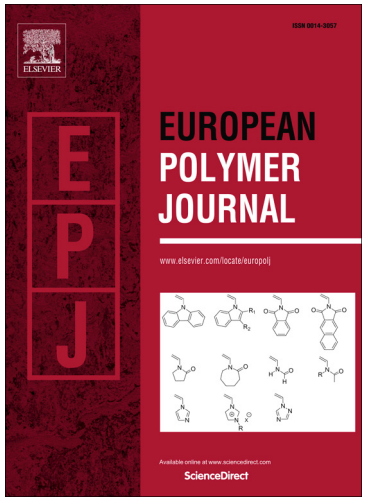

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Inherent antibacterial activity and in vitro biocompatibility of hydrophilic polymer film containing chemically anchored sulfadiazine moieties

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Abstract

Microbial colonisation of synthetic materials is a great concern in many fields, e.g., in implant surgery and medical devices; therefore biocompatible hydrophilic organic materials with inherent antimicrobial properties are of current research interest. In this work, we describe the preparation of antibacterial and biocompatible polymeric film based on *N*-vinyl-2-pyrrolidinone (VP) and 2-hydroxyethyl acrylate (HEA), using ethyleneglycol dimethacrylate (EGDMA), and synthetic acrylic monomer containing sulfadiazine

chemically anchored. The synthesized polyvinylpyrrolidone (PVP)-based films were characterized by different techniques (^{1}H and ^{13}C NMR, ATR-FTIR, SEM, and TGA). In this study, the biophysical responses of bacteria and L929 cells towards the prepared materials as model device surfaces were evaluated. The membrane that contains the anchored sulfadiazine moiety showed excellent antibacterial activity against *Escherichia coli* as well as good biocompatibility. Based on the experimental results, this material is a good candidate for medical applications as biomaterial.

***Keywords*:** PVP, sulfadiazine, antibacterial polymer, biocompatible.

1. Introduction

The microbial colonisation of synthetic materials is one of the main concerns of several industries such as water treatment, general consumer goods and biomedical implants and devices, to name but a few [1,2]. This is especially concerning when it comes to implant surgery and medical devices where there has to be a balance between antibacterial properties and biocompatibility [3]. Extensive research has been conducted on antibacterial properties of materials [2,4,5]. However the threat of emerging and widespread bacterial resistance to antibiotics [6] which are by far the most commonly used antimicrobial pharmaceutical agents, is endangering their efficacy [7]. In addition to this, new drug development by the pharmaceutical industry has been stalled [8]. Therefore, the lack of new alternatives to treat bacterial infections has become a major global concern that needs to be addressed.

Although "ESKAPE" bacteria are currently studied due to their importance in hospital-acquired infections, *Escherichia coli* is especially prevalent as it is the cause of more Gram-negative infections than *Klebsiella pneumonia* and *Enterobacter* species combined [9]. *E. coli* is one of the most frequently isolated pathogens in different types of medical devices

and surgical sites [10] and can induce the growth and virulence in pigmenting anaerobes, and consequently, these also colonise the medical devices [11].

There is a need to develop new materials that are both an alternative to existing antimicrobial treatments and also address the risk of infection from bacteria outside the "ESKAPE" group such as *E. coli*. Once the etiologic pathogen has been identified and/or antimicrobial susceptibility data are available, every attempt should be made to narrow the antibiotic spectrum. This is a critically important component of antibiotic therapy because it can reduce cost and toxicity and prevent the emergence of antimicrobial resistance in the community [6]. One of the methods that have been implemented is the incorporation of different chemical compounds such as antibiotics to the designed materials [12-14].

In this work, we prepared biocompatible materials based on a copolymer composed of *N*-vinyl-2-pyrrolidone (VP) and 2-hydroxyethyl acrylate (HEA). VP and HEA are broadly applied in cosmetics, foods, adhesives, textiles and specifically as biomaterials [15-17]. In addition to this, sulfadiazine (SDZ) was incorporated into the materials to provide them with antibacterial activity of narrow spectrum. This antibiotic is used in certain therapeutic fields [18] and is on the World Health Organization's list of essential medicines [19].

In order to improve the antibacterial efficiency whilst maintaining the biocompatibility, a method to incorporate the antibiotic has been proposed. A sulfadiazine moiety (acrylic monomer containing sulfadiazine) was synthesised and chemically anchored to the co-polymeric film to avoid the migration and residual toxicity of the antibiotic [4,20,21]. Also, this anchorage of the sulfadiazine co-monomer provides the possibility of sustained release over a prolonged period compared to the native monomer of the drug dispersed in the film [4,22].

In summary, biocompatible films with improved antibacterial activity were prepared and characterized by different techniques. Also, their antibacterial properties and biocompatibility were assessed.

2. Materials and methods

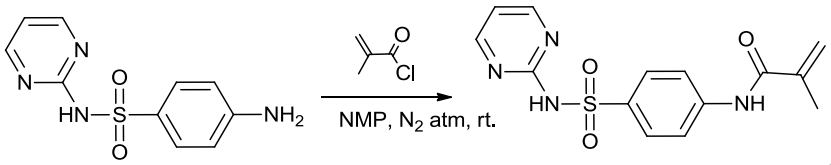
2.1. Materials for the synthesis of monomer and films

All compounds and solvents were commercially available and used as received: *N*-vinyl-2-pyrrolidinone (VP) (Aldrich, 99%), 2-hydroxyethyl acrylate (HEA) (Aldrich, 96%), ethylene glycol dimethacrylate (EGDMA) (Aldrich, 98%), methacryloyl chloride (Fluka, 97%), 1-methyl-2-pyrrolidone (NMP) (Aldrich, 99%) and sulfadiazine (SDZ) (4-amino-*N*-(pyrimidin-2-yl)benzenesulfonamide) (Alfa Aesar, 99%). Azo-bis-isobutyronitrile (AIBN) (Aldrich, 99%) was recrystallized twice from methanol.

2.2. Synthesis of sulfadiazine-containing acrylic monomer

The synthesis of sulfadiazine-containing acrylic monomer *N*-(4-(*N*-(pyrimidin-2-yl)sulfamoyl)phenyl)methacrylamide (SDZ-MA) was performed as follows: 5g (20mmol) of sulfadiazine (SDZ) was dissolved in 20 mL of NMP in a round-bottom flask under nitrogen. Afterwards, 2.3 mL (24mmol) of methacryloyl chloride was added dropwise. It was stirred at room temperature for 4 h. The solution was added dropwise to water (500 mL) under vigorous stirring, yielding a white solid product that was obtained after filtering. Then it was washed three times with 15 mL of water and dried under vacuum at 40°C overnight. Yield: 5.7 g, 90%).^{1}H-NMR (δ_H ppm) (400 MHz, DMSO- d_6, Me_4Si):10.17 (s, 1H), 8.49 (d, 4.9 Hz, 2H), 7.98 – 7.83 (m, 4H), 7.02 (t, 4.9 Hz, 1H), 5.85 (s, 1H), 5.55 (s, 1H), 1.93 (s, 3H).^{13}C-NMR (δ_C ppm) (100.6 MHz, DMSO-d_6, Me_4Si):167.35, 158.38, 156.99, 143.16, 140.01, 134.30, 128.67, 120.98, 119.46, 115.83, 18.65.FT-IR (Wavenumbers, cm^{-1}): ν_{N-H}:

3401, $\nu_{C=O}$ (Amide I): 1695. EI-HRMS m/z: calcd for $C_{14}H_{14}N_4O_3S$ 318.0787, found 318.0769. The synthesis is shown in Scheme 1.



SDZ-MA

Scheme 1. Synthesis of sulfadiazine-containing acrylic monomer *N*-(4-(*N*-(pyrimidin-2-yl)sulfamoyl)phenyl)methacrylamide (SDZ-MA).

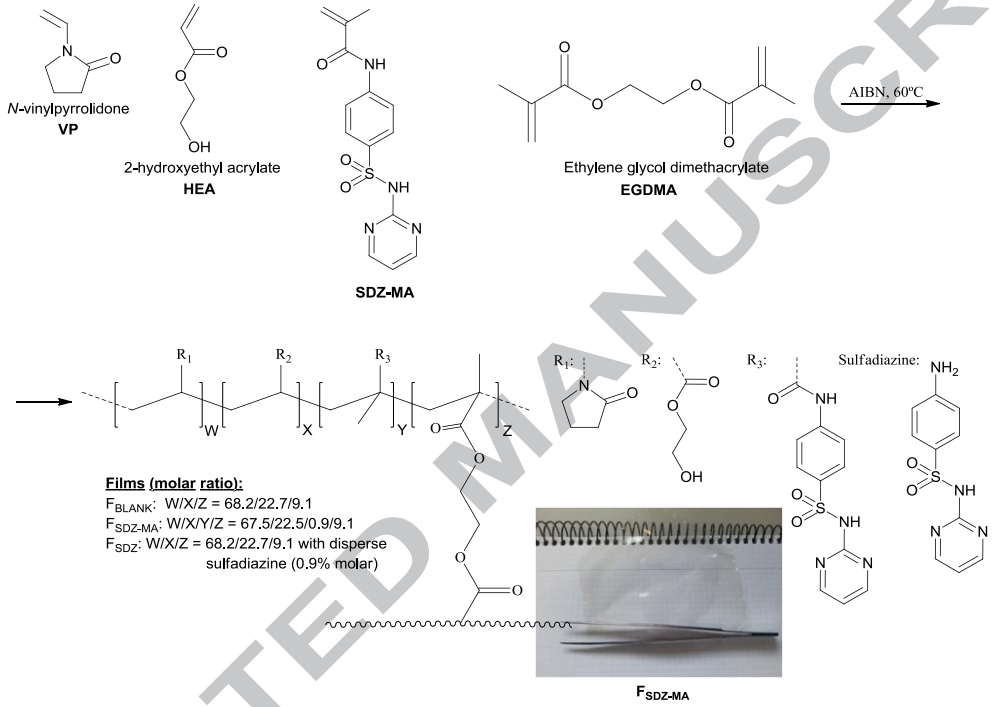
2.3. Film preparation

The film F_{BLANK} was prepared by radical polymerization of VP and HEA, using ethylene glycol dimethacrylate (EGDMA) as the cross-linking agent, with a feed co-monomer molar ratio of 68.2/22.7/9.1 for VP/HEA/EGDMA, and AIBN (1 wt %) as a thermal radical initiator. Afterwards, the thermally initiated bulk radical polymerization reaction was carried out in a silanized glass mould of 100 μm thickness under an oxygen-free atmosphere at 60°C overnight.

The film $F_{SDZ\text{-}MA}$, which contains sulfadiazine moiety SDZ-MA (acrylic monomer with sulfadiazine) was prepared by adding 1% (mole) of this co-monomer to the mixture of monomers used to prepare the blank film F_{BLANK}. Thus, the feed co-monomer molar ratio was of 67.5/22.5/0.9/9.1 for VP/HEA/SDZ-MA/EGDMA respectively and AIBN (1 wt %) as a thermal radical initiator. The polymerization was carried out as described for F_{BLANK}.

The film F_{SDZ} was prepared as described for F_{BLANK} by dispersing SDZ in the mixture of monomers VP/HEA/EGDMA (molar ratio of 68.2/22.7/9.1) using AIBN (1 wt %) as thermal radical initiator. The molar ratio of SDZ to the other monomers was 0.9%.

The film preparation and compositions are detailed in Scheme 2.



Scheme 2. Films F_{BLANK} and $F_{SDZ\text{-}MA}$ preparation and composition (inset: picture of film $F_{SDZ\text{-}MA}$). F_{SDZ} was prepared similarly to F_{BLANK} by dispersing sulfadiazine in the mixture of monomers.

2.4. Characterization techniques and procedures

Elemental analysis was obtained using a LECO CHNS-932 analyser and a VTF-900 equipped with an ultra-microbalance SARTORIUS M2P (accuracy ± 0.001 mg).

^{1}H and ^{13}C NMR spectra were recorded at 399.92 and 100.57 MHz, respectively, on a Varian Unity Inova 400 MHz spectrometer. All NMR data were recorded at 25°C in

deuterated dimethyl sulfoxide (DMSO-d_6), and both ^{1}H and ^{13}C chemical shifts were referenced to the residual signal of this solvent.

HRMS spectra were obtained in a hybrid spectrometer API QSTAR® XL Hybrid system (Applied, Biosystems) using ESI of high resolution with DMSO as the solvent.

FTIR spectra were obtained using a PERKIN ELMER BX-FTIR spectrometer coupled with an Attenuated Total Reflectance (ATR) accessory, MIRacle™ ATR from PIKE Technologies and interferograms were obtained from 32 scans.

Thermogravimetric analysis (TGA) data were recorded using 4-5 mg of sample under a nitrogen atmosphere on a TA Instrument Q50 TGA analyser at a scan rate of 10°C min^{-1}.

Wettability of the membranes was determined by contact angle measurements (CA). The sessile drop technique was selected to estimate the advanced contact angles for deionized/Millipore water using CAM200 KSV equipment. Each θ was calculated by a minimum of five independent measurements at room temperature.

The swelling behaviour of the films was determined by immersion in deionized water at 20±0.1°C. The water-swelling percentage (WSP) was calculated by weighing the dry membrane (ω_d) and the swollen membrane (ω_s) using an electronic balance (Sartorius, BP 210S, d = 0.1 mg). The WSP was calculated using the following formula: $[(\omega_s-\omega_d)/\omega_d] \times 100$.

2.5.Antibacterial activity

2.5.1. *Disk diffusion method*

The antibacterial activity of the polymeric films was tested against a Gram-negative bacteria, *Escherichia coli* DSM-No 301, that was used in previous studies [23]. The method

was carried out in a Trypticase Soy Agar (TSA, Scharlau) medium petri dish. The membranes were cut in 6 mm Ø disks and placed on 10^8 CFU·mL^{-1} bacterial concentration agar plates which were incubated at 37ºC for 24h. Silver nitrate and distilled water were used as positive and negative control, respectively [24]. The results were obtained by measuring the diameter of zones of inhibition. The obtained data are expressed as means and standard deviations of two experiments carried out in triplicate. The

2.5.2. Bioassay procedure and indirect impedance technique

Bacterial growth was conducted at 37ºC in bioreactors of 7 mL of capacity filled with 1 g of sterile silica and 1.5 mL of bacterial suspensions of *E. coli* in minimal medium (MM) supplemented with 5 g·L^{-1} of glucose at a concentration of $2.5 \cdot 10^7$ cells·mL^{-1}, prepared as described in previous work [25]. Afterwards, disks of F_{BLANK}, F_{SDZ} and $F_{SDZ\text{-}MA}$ (5 mg) were added to the medium. Also, silver nitrate was used as positive control. These bioreactors were introduced in 20-mL disposable cylindrical cells charged with 1.5 mL of 2 g·L^{-1} KOH aqueous solution and provided with electrodes to measure impedance on a Bac-Trac 4300 (SY-LAB Geräte GmbH, Neupurkerdorf, Austria). The device monitors the relative change in the initial impedance value of KOH solution, which is converted to concentration of carbon dioxide by a calibration curve of impedance variation versus concentration of CO_2. The experimental device and procedure have been described in previous works [26, 27].

2.6. Biocompatibility studies

2.6.1. Culture of cells

L929 mouse fibroblasts (Sigma Aldrich) were chosen as a reference cell line to study the biocompatibility of biomaterials [25, 28]. L929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS),

(BioWhittaker, Europe, Belgium), 2mM L-glutamine, penicillin (100 $IU \cdot mL^{-1}$) and streptomycin (100 $\mu g \cdot mL^{-1}$) (Sigma Aldrich), under 5% CO_2 atmosphere and at 37ºC for different times. Before using, the membranes were sterilized by UV irradiation for 1h.Tissue culture polystyrene (TCP) was used as control.

2.6.2. Cytotoxicity assay

Cytotoxicity of the membranes was assessed by the reduction of the MTT reagent (3-[4,5-dimethyl-thiazol-2-yl]- 2,5-diphenyltetrazolium bromide) (Sigma Aldrich) to formazan, following the method proposed by Tada *et al.* [29]. Cells were seeded on the membranes disks previously placed in a 24-well culture plate at a density of $1 \cdot 10^5$ $cells \cdot mL^{-1}$. The samples were incubated for different times under the mentioned conditions. The absorbance at 590 nm was measured using a LabTech LT-4000 spectrophotometer.

2.6.3. Cell adhesion studies

Adhesion of the L929 cells was observed using a FeiNova NanoSEM 230scanning electron microscope. L929 cells were seeded on the different membranes with a density of $2 \cdot 10^5$ cells mL^{-1} using a 24-well culture plate, which was incubated for 24 h. The membranes were washed twice with PBS and fixed with 2.5% glutaraldehyde for 60 min. After washing, the samples were dehydrated by slow water replacement using series of ethanol solutions (30%, 50%, 70% and 90%) for 15 min and finally dehydrating in absolute ethanol for 30 min, allowing samples to dry at room temperature. The samples were metalized to perform the micrographs.

Also, L929 cells attachment to the membranes was quantified by counting the cells using a Neubauer haemocytometer. The cell culture media was collected along with two washes of

PBS to count the unattached cells. The percent cell attachment was calculated as described in the literature [30].

2.6.4. Statistical analysis

Analysis of variance test (ANOVA) was performed to make the statistical comparisons by using the Statistical Package for the Social Sciences version 21 (SPSS® Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Monomer synthesis and films preparation

The sulfadiazine-containing acrylic monomer SDZ-MA was straightforward and inexpensively prepared in one high-yield synthetic step from widely available chemicals using the proven organic reaction (Scheme 1). SDZ-MA was characterised by FTIR and ^{1}H and ^{13}C NMR spectroscopy (Figure 1). The obtained spectra of the monomer fully confirmed the chemical structure of all the products.

The polymer dense membranes, or films, were prepared conventionally by thermally initiated radical polymerization of the co-monomers. The co-monomers and initiator solutions were injected in a closed flat glass mould with controlled thickness under an oxygen-free atmosphere. This is an optimised procedure for preparing membrane films used in sensing applications [31-33]. The structure of the copolymer network of the membrane is depicted in Scheme 2 along with a digital picture of one of the membranes. The films showed good optical properties, were highly manageable (see picture, Scheme 2) and also presented reasonable thermal resistance (Table 1). The acrylic monomer SDZ-MA and the dispersed SZD were incorporated to the dense cross-linked membranes at a molar

percentage of 0.9%, meaning that its presence in the membrane network has a negligible impact in the material properties and low economical impact.

Table 1. Thickness, hydrophilicity (water swelling percentage – WSP - and contact angle -θ-) and thermal resistance (temperature at which 5% weight loss is observed - T_5 -) of films.

Film	Thickness (μm)	*WSP* (%)	θ (°)	T_5
F_{BLANK}	114 ± 0.7	47 ± 1	60.2 ± 0.8	322
F_{SDZ}	108 ± 0.9	86 ± 1	36.0 ± 0.9	314
$F_{SDZ\text{-}MA}$	115 ± 1.0	58 ± 1	42.3 ± 1.1	171

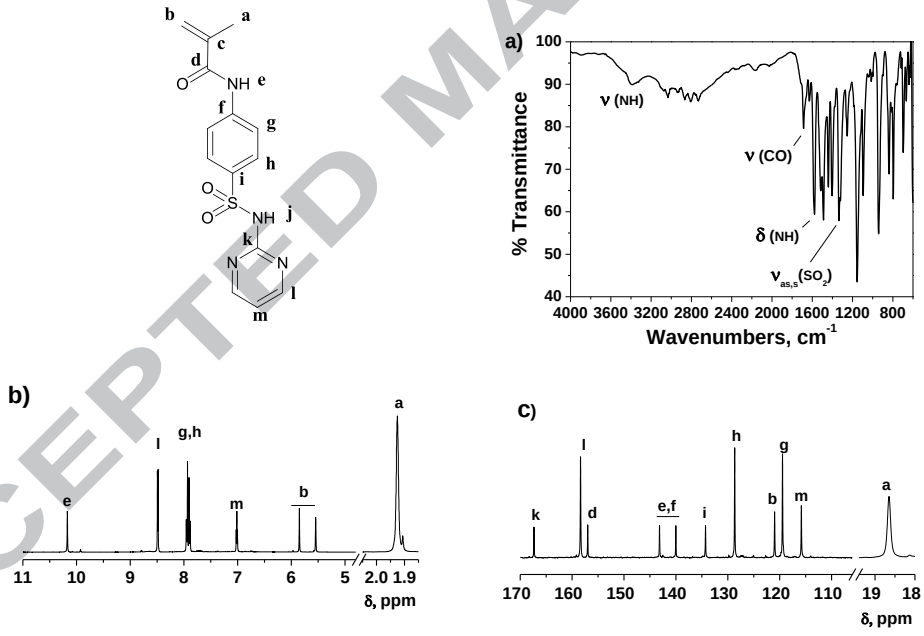


Figure 1. Characterization of SDZ-MA: a) FTIR; b) ^{1}H NMR; c) ^{13}C NMR. NMR solvent: DMSO-d_6.

3.2. Film hydrophilicity

The hydrophilic character is a key parameter in a number of biomedical applications of biomaterials. The hydrophilicity of the films was assessed by determining the water affinity of the surface of the materials by using the contact angle technique, rendering the contact angle (θ).The overall hydrophilicity of the films was analysed considering the water uptake upon dipping the films in pure water until water regains equilibrium, thus obtaining the water swelling percentage.

A surface is considered to be hydrophilic if the contact angle (θ) is below 90º [34]. Accordingly, the surfaces of the films (Table 1) are highly hydrophilic (θ <61º). The hydrophilic character of the surfaces of F_{SDZ} and $F_{SDZ\text{-}MA}$ is higher than that of F_{BLANK} due to the dispersion or the anchorage of moieties with sulfonamide groups that have a high affinity for water molecules. F_{SDZ} shows higher hydrophilic nature due to the free primary amine group. It has higher mobility arisen from the dispersion nature of the SDZ. In comparison to this, $F_{SDZ\text{-}MA}$ has a mobility restriction imposed by the chemical anchorage of the sulfadiazine residue. The contact angle data are in full agreement with the WSP. The WSP of F_{BLANK} shows the hydrophilic nature of the films in general, however there was a significant increase in the water affinity for F_{SDZ} and $F_{SDZ\text{-}MA}$. This follows the trend observed in the wettability results of the three films.

3.3. Antibacterial activity

The antibacterial activity of the materials based on the copolymer composed of VP and HEA was evaluated by performing the disk diffusion test and indirect impedance technique. The

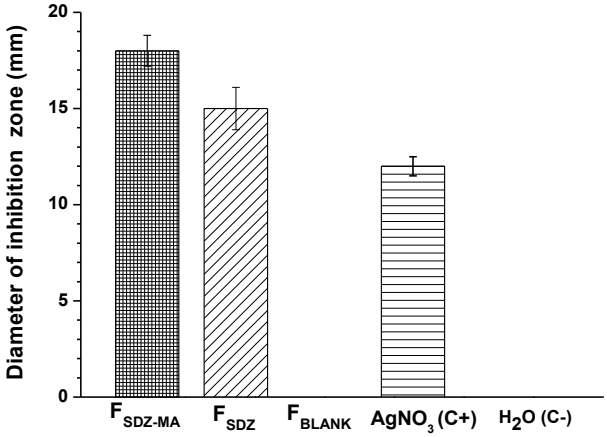
results obtained for the synthesised membranes F_{BLANK}, F_{SDZ} and $F_{SDZ\text{-}MA}$ are shown in Figure 2.

In the disk diffusion test, F_{BLANK} and the film containing dispersed sulfadiazine F_{SDZ} formed halos of 0 and 15 mm in diameter, respectively (Figure 2A and 2B). These results demonstrated that they were not effective in inhibiting the growth of *E. coli*, because as it is shown in the literature, for the sulfonamides any values below 16 mm in diameter indicate that the compounds are not antibacterial [35]. The positive control $AgNO_3$ produced a halo of 12 mm confirming that was also ineffective against this bacteria. However, the zone of inhibition for $F_{SDZ\text{-}MA}$ had a value of 18 mm, which demonstrate that this material has high antibacterial activity. At this point it is important to underline the selectivity of the material toward the inhibition of the growth of *E. coli*, diffusion test showed that the membranes did not have an antibacterial effect on a number of Gram-negative and Gram-positive bacteria.

On the other hand, the antibacterial activity of the membranes was monitored by measurements of indirect impedance. The production of carbon dioxide during the bacteria metabolism was then correlated to the degree of glucose biodegradation in a period of 24 h, and the profiles are shown in Figure 2C. Both control and F_{BLANK} presented 50% biodegradation of glucose which confirms that this blank membrane has no antibacterial capability against *E. coli*. In the case of F_{SDZ} and $AgNO_3$, the percentages reached 23 and 28%, respectively, showing similar biodegradation profiles but insufficient to be considered as antibacterial. However, for $F_{SDZ\text{-}MA}$, the biodegradation decreased drastically reaching only 10% biodegradation. According to the results obtained in this assay as well as in the disk diffusion test, these confirmed that $F_{SDZ\text{-}MA}$ has a remarkable antibacterial activity.

This feature of $F_{SDZ\text{-}MA}$ can be caused by the enzymatic cleavage of the chemical linkage of sulfadiazine moiety [22, 35]. The breaking of this linkage allows the controlled release of the antibiotic in FSDZ-MA which does not occur in FSDZ. This leads to a slower release of the bioactive compound and prolonged activity [22]. The design of this material allows to target *E. coli* specifically by avoiding the use of antibiotics that could cause resistances. The use of $F_{SDZ\text{-}MA}$ could play an important role in medical device contaminations in which *E.coli* [10] is one of the most frequently isolated pathogens. Impeding the presence of this bacteria is also essential due to its role in inducing other bacteria to also colonise the medical devices [11].

A



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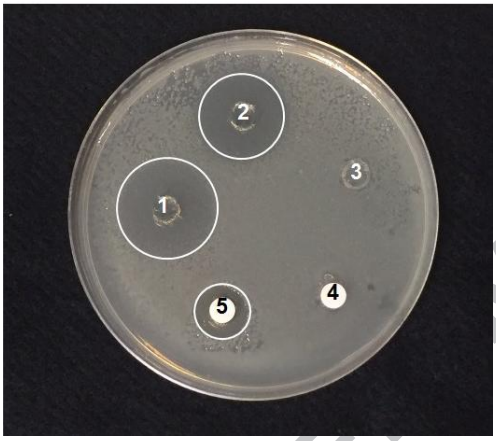

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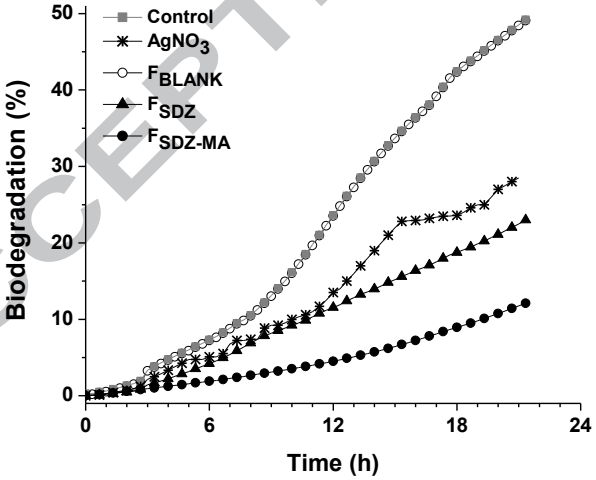

Figure 2. Antibacterial activity of the films against *E. coli* by disk diffusion test: A) Diameter of inhibition zones. Data are expressed as means ± SD (n=6).B) Zones of growth inhibition on the agar plate: 1- F_{SDZ-MA}, 2- F_{SDZ}, 3- $F_{BLANK,}$ 4- Distilled water (negative control), 5- $AgNO_3$ (positive control). The white circles show the zones of inhibition against *Escherichia coli*. C) Glucose biodegradation profiles during 24 hours in the presence of *Escherichia coli*.

3.4. Biocompatibility studies

The biocompatibility of the materials based on PVP-HEA copolymer was evaluated by cytotoxicity and cell adhesion using L929 cell line.

The cytotoxicity of the materials was studied by MTT assay for 3 days. Mitochondrial redox activity of L929 fibroblasts on the membranes was evaluated by reduction of MTT to formazan [36]. The results showed a good mitochondrial activity for the membranes F_{BLANK}, F_{SDZ} and $F_{SDZ\text{-}MA}$ where no significant differences were observed in respect to the control ($p>0.05$) (Figure 3).

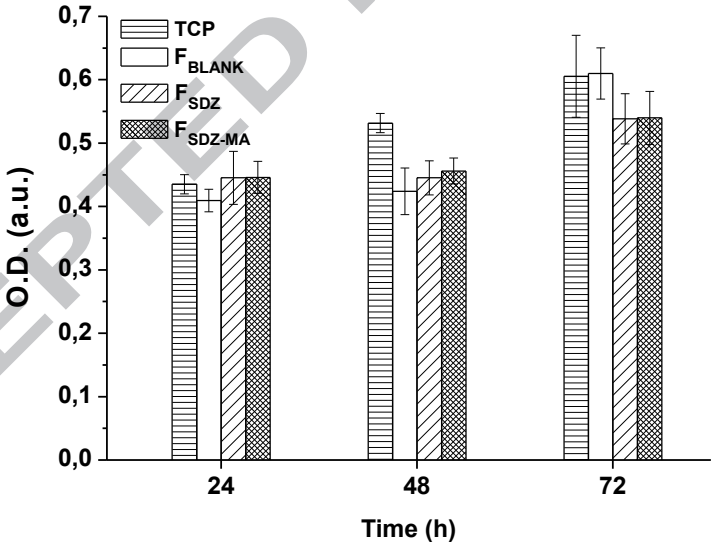

Figure 3. MTT assay optical density (O.D.) for the L929 on the membranes after different culture times. Data are expressed as means ± SD (n=9). Statistical significance: *$p<0.05$.

Also, the adhesion of L929 cells was studied by SEM and cell counting (Figure 4). As shown in SEM micrographs, the adhesion of the fibroblasts to the membranes F_{BLANK}, F_{SDZ}

and $F_{SDZ\text{-}MA}$ was practically null (Figure 4A).These results correspond to the data obtained by cell counting (Figure 4B). The control showed 85% of cell attachment whereas the cell adhesion to the surface of the membranes was significantly impeded only reaching values below 2% ($p<0.05$).This null cell adhesion is due to the high hydrophilicity of the membranes. These prevent the adsorption of cell adhesion-mediating proteins. [37]. Inhibiting protein adsorption at the polymer surface is a distinctive character of PVP-based biomaterials[38, 39]. This is important as the protein deposition can affect the functions of the medical devices, cause clinical complications or shorten the lifespan of the polymer. The null cytotoxicity and null protein fouling to the materials confirmed their good biocompatibility as biomedical devices.

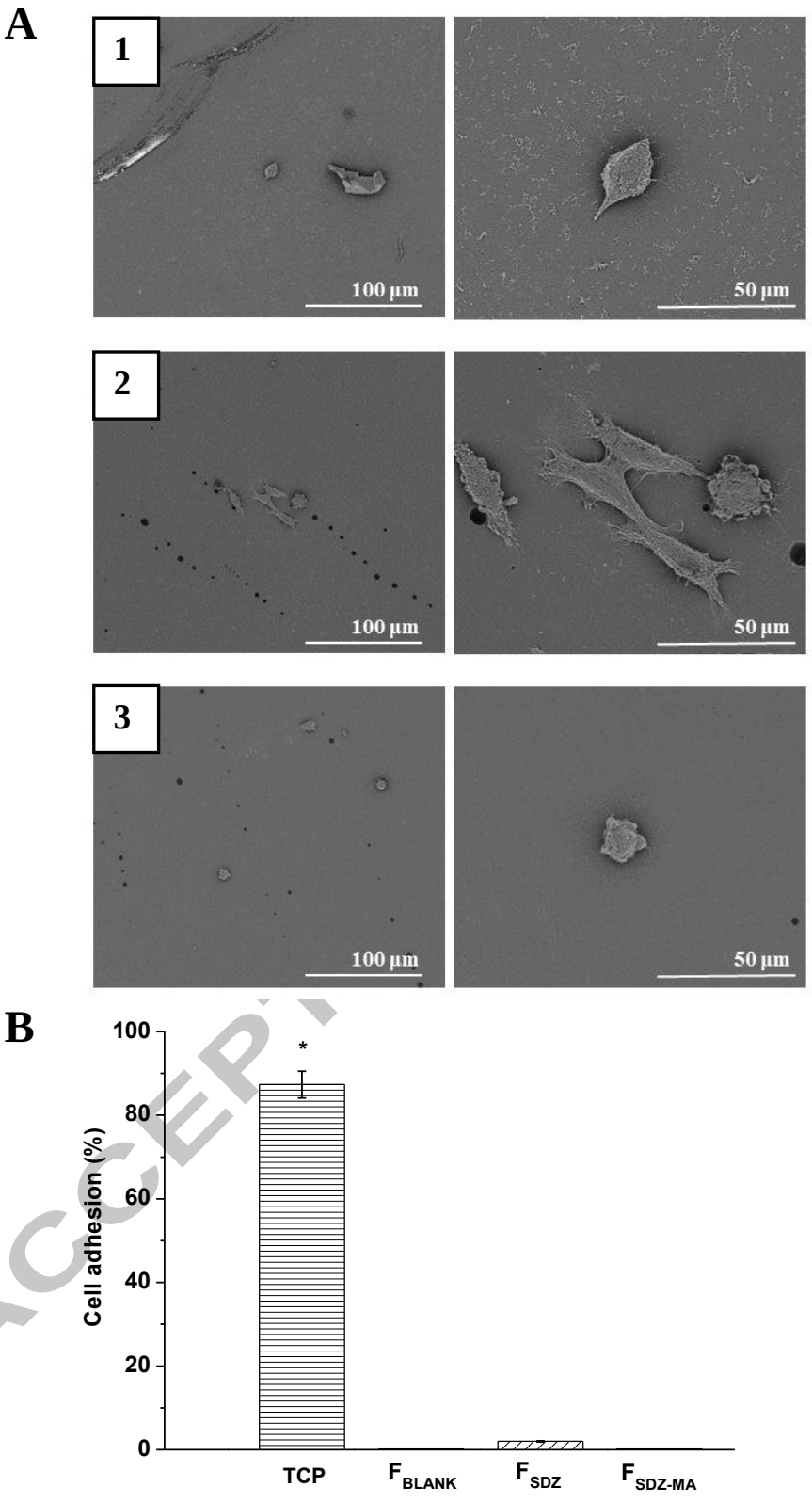

Figure 4. A) SEM micrographs (magnification, left: 1000x; right: 4000x) of the cell adhesion on the films: 1- F_{BLANK}; 2- F_{SDZ}; 3- $F_{SDZ\text{-}MA}$. B) Percentages of cell adhesion after 24h of incubation. Data are expressed as means ± SD (n=9). Statistical significance: *$p<0.05$.

4. Conclusions

In summary, two membranes based on *N*-vinyl-2-pyrrolidinone (VP) and 2-hydroxyethyl acrylate (HEA) were synthesised and tested for antibacterial activity and biocompatibility. A blank membrane F_{BLANK} was prepared by radical polymerization using ethylene glycol dimethacrylate (EGDMA). This film was used as a base material to be modified by dispersing a pharmaceutical compound (SDZ) or anchoring a new synthesised sulfadiazine moiety (SDZ-MA). According to the biocompatibility studies, the two new films F_{SDZ} and $F_{SDZ\text{-}MA}$ were not cytotoxic for L929 cells and impeded protein fouling because of the high hydrophilicity of their surfaces. However, the antibacterial activity tests confirmed that only $F_{SDZ\text{-}MA}$ was effective against *E. coli*. This could be explained by the chemical bond of the sulfadiazine moiety that allowed a slow excretion and more prolonged activity of the sulfadiazine. Therefore, this demonstrates that the chemical anchorage performed on $F_{SDZ\text{-}MA}$ improved the antibacterial activity of the native sulfadiazine while maintaining the biocompatibility properties of the original polymer, PVP. This makes this new membrane appropriate for specific biomedical applications and especially optimal against *E.coli* colonization.

Acknowledgements

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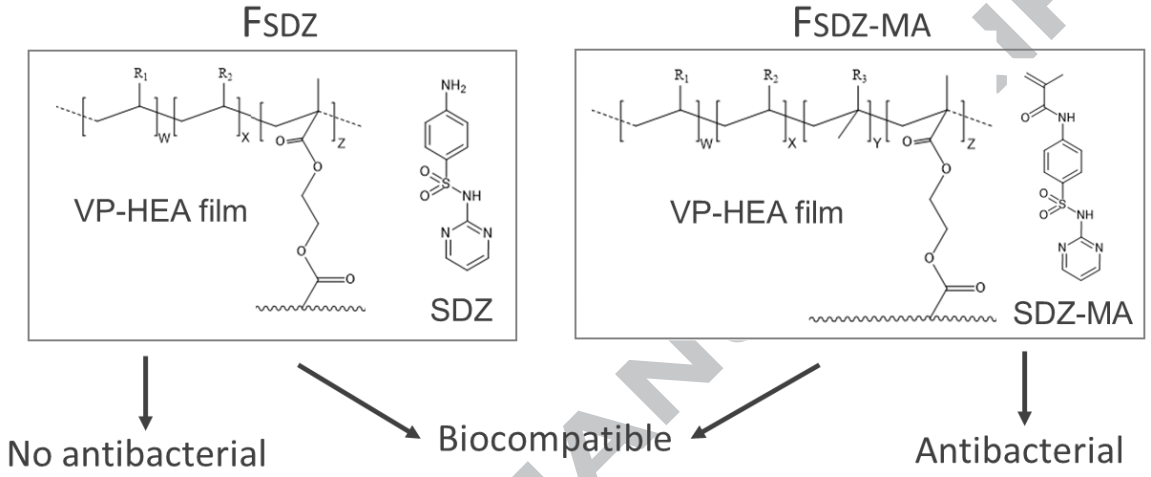
Graphical abstract



Highlights (85 characters maximum including spaces)

- Films based on VP and HEA were prepared by radical polymerization.
- The synthesised sulfadiazine moiety (SDZ-MA) was chemically anchored to the film.
- The film ($F_{SDZ\text{-}MA}$) presented improved antibacterial activity and good biocompatibility.