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Bacterial cellulose membranes as transdermal delivery systems for diclofenac: *In vitro* dissolution and permeation studies

Nuno H.C.S. Silva[a], Artur Filipe Rodrigues[a], Isabel F. Almeida[b], Paulo C. Costa[b], Catarina Rosado[c], Carlos Pascoal Neto[a], Armando J.D. Silvestre[a], Carmen S.R. Freire[a,*]

[a] CICECO, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
[b] Laboratory of Pharmaceutical Technology, Department of Drug Sciences, Faculty of Pharmacy, University of Porto, Portugal
[c] CBIOS, UDE – Experimental Dermatology Unit, Universidade Lusófona, Campo Grande 376, 1749-024 Lisbon, Portugal

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ABSTRACT

Bacterial cellulose (BC) membranes were explored as novel nanostructured transdermal delivery systems for diclofenac sodium salt (a typical non-steroidal anti-inflammatory drug). Diclofenac sodium salt loaded BC membranes were prepared through a simple methodology, using glycerol as plasticizer, and characterized in terms of structure, morphology and swelling behavior. The membranes were very homogeneous, quite flexible and presented a considerably higher swelling behavior when compared with pure BC. *In vitro* diffusion studies with Franz cells, were conducted using human epidermal membranes, and showed that the incorporation of diclofenac in BC membranes provided similar permeation rates to those obtained with commercial patches and substantially lower than those observed with a commercial gel. This release profile together with the ease of application and the simple preparation and assembly of the drug-loaded membranes clearly indicates the enormous potentialities of using BC membranes for transdermal administration of diclofenac.

1. Introduction

Diclofenac 2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid, is a non-steroidal anti-inflammatory drug, commonly used to relief pain and inflammation in several clinical short-term situations, such as sprains and strains, surgeries and dental work (DiPiro et al., 2008). It is also frequently prescribed for long term treatment of rheumatoid arthritis and osteoarthritis (Fuller & Roth, 2011; Laine et al., 2007).

Oral administration, using conventional tablets, implies that the drug is subjected to the first pass metabolism in the liver, leaving only about 50% of the drug available systemically (Goodman & Gilman, 2008; Hui et al., 1998). On the other hand, although several oral controlled-release pharmaceutical dosage forms have been developed, diclofenac is known to induce ulceration and bleeding of the intestinal walls (Heyneman, Lawless-Liday, & Wall, 2000). To avoid these adverse effects, alternative routes of administration, namely topical administration systems as gels and transdermal patches, are also commercialized. The use of topical or transdermal patches is particularly straightforward because of the simplicity of application, dosage uniformity and noninvasive nature. They enable a significant by-pass of pre-systemic metabolism, either by the gastrointestinal tract or by the liver, thereby increasing drug bioavailability. Additionally, once steady-state is reached, drug levels can be maintained in the systemic circulation within the therapeutic window, which enables a prolonged duration of action after a single application and a reduction in the frequency of dosing (Delgado-Charro & Guy, 2001).

Transdermal patches are normally composed of superimposed layers of different materials that serve as reservoirs of the drugs and/or control their release usage. Several synthetic polymers such as poly(ethyl methacrylate) (PEMA), poly(methyl methacrylate) (PMMA) and polyvinylpyrrolidine (PVP) are commonly used in this type of medical devices (Arora & Mukherjee, 2002; Fang, Sung, Lin, & Fang, 1999). More recently, biopolymers (or their derivatives), because of their renewable character, biodegradability, non-toxicity and specific properties, have also been considered as promising materials for the development of drug delivery systems. For example, cellulose derivatives as hydroxypropyl cellulose, ethylcellulose and methyl cellulose are widely used in several diclofenac topical formulations (El-Sousi et al., 2013; Fang et al., 1999). Acrylic acid grafted guargum-nanosilica based membranes (Giri et al., 2013) and blends of amylase-pectin (Soares, de Castro,

* Corresponding author at: CICECO, Department of Chemistry, University of Aveiro, Campus de Santiago, 3810-193 Aveiro, Portugal. Tel.: +351 234 370 604.
E-mail address: cfreire@ua.pt (C.S.R. Freire).

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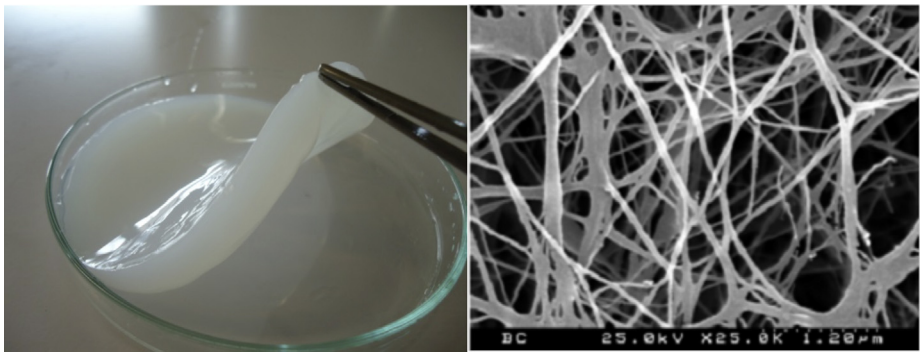

Fig. 1. Visual image of a bacterial cellulose (BC) membrane and its tridimensional nanofibrillar structure displayed by SEM analysis (freeze-dried sample produced in our laboratory).

Cury, & Evangelista, 2013) were also developed and reported as promising systems for the transdermal release of diclofenac.

Cellulose is the most abundant biopolymer and is mainly biosynthesized by plants; however, several bacteria can also produce an extra-cellular form of cellulose, commonly known as bacterial cellulose (BC). BC is produced in the form of highly swollen membranes (>90% water content) (Fig. 1), in the culture medium/air interface (Gatenholm & Klemm, 2010; Iguchi, Yamanaka, & Budhiono, 2000; Klemm, Heublein, Fink, & Bohn, 2005). The unique physical and mechanical properties, arising from its tridimensional nanofibrillar structure (Fig. 1), as well as its high purity and biocompatibility engendered considerable interest on this material in the biomedical field (Czaja, Young, Kawecki, & Brown, 2007) specifically as wound healing membranes (Lina, Yue, Jin, & Guang, 2011) scaffolds for tissue engineering (Mori, Nakai, Enomoto, Uchio, & Yoshino, 2011), artificial blood vessels (Klemm, Schumann, Udhardt, & Marsch, 2001) and drug delivery systems (Müller et al., 2013; Silva et al., 2014; Trovatti et al., 2011, 2012). In the latter case, BC nanostructured membranes could be particularly advantageous in the design of transdermal drug delivery systems because of the straightforwardness and effectiveness of preparation of the drug loaded BC membranes and the fact that they are only composed of a single layer. Their ability to absorb exudates and to adhere to irregular skin surfaces, along with their conformability, are additional issues that are crucial for several clinical situations.

Recent studies carried out in our group indicated that BC can be successfully applied to modulate the bioavailability of lidocaine and ibuprofen (Trovatti et al., 2012) and caffeine (Silva et al., 2014) for dermal administration, furthermore BC *in vivo* skin compatibility for dermal applications has been recently demonstrated by a clinical study (Almeida et al., 2013). Müller et al. (2013) also reported the use of pure BC membranes for topical delivery of active proteins.

In this sense, the aim of this study was to further investigate the potential of BC membranes as transdermal systems for the delivery of diclofenac sodium salt (hereafter referred simply as diclofenac). The preparation of the BC-diclofenac membranes was developed and optimized aiming to obtain a homogeneous and reproducible material. The *in vitro* dissolution and permeation through human epidermis in BC was then studied and compared with two commercial formulations (gel and patch), in order to assess its therapeutic applicability and feasibility.

2. Materials and methods

2.1. Materials

Diclofenac sodium salt (99%) and glycerol (99.5%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Solvents and other reagents were of analytical grade. BC membranes (about 99% water content) were produced using the bacteria *Gluconacetobacter sacchari* (Trovatti, Serafim, Freire, Silvestre, & Neto, 2011) and conventional culture media conditions (Hestrin & Schramm, 1954).

Commercially available formulations were used for comparative purposes. Diclofenac Sandoz®, 1% (w/w) diclofenac sodium salt gel formulation (Sandoz Farmacêutica Lda, Sintra, Portugal) and Olfen® 0.5% (w/w) diclofenac sodium salt patch formulation (Mepha Lda, Porto Salvo, Portugal).

2.2. Preparation of BC-diclofenac membranes

Wet 7 cm × 0.8 cm circular BC membranes (~160 mg dry weight) were weighted, and handily compressed between two acrylic plates at room temperature to remove 50–60% of their water content. Drained BC membranes were then soaked in 8 ml of an aqueous buffered solution (pH 7.4) of diclofenac sodium salt (1% or 0.5%) and glycerol 5%, during 48 h at room temperature to assure complete absorption of the drug. After the total absorption of the solution, the BC membranes were placed over Petri dishes and dried at 40 °C in a ventilated oven for 16 h. BC-glycerol (BC-glyc) membranes were prepared according to this method without adding diclofenac. And pure BC membranes were prepared by drying drained BC membranes at 40 °C in a ventilated oven for 16 h. All membranes were kept in a desiccator until their use. The identification of all samples prepared is summarized in Table 1.

2.3. Characterization of BC-diclofenac membranes

BC and BC-diclofenac dried membranes were characterized in terms of structure, surface morphology, tensile mechanical properties, and swelling behavior.

Fourier transform infrared (FTIR) spectra were obtained in a Perkin-Elmer FTIR system spectrometer equipped with a single horizontal Golden Gate ATR cell. Thirty-two scans were acquired in the 4000–600 cm^{-1} range with a resolution of 4 cm^{-1}. 4 samples of each membrane were analyzed in different points.

SEM micrographs of the BC and BC-diclofenac membranes surfaces were obtained on HR-FESEM SU-70 Hitachi equipment operating at 15 kV. Samples were placed in a steel support and

Table 1
Identification of the samples prepared in this study.

Sample	Diclofenac sodium (%)[a]	Glycerol (%)[a]
BC	–	–
BC-glyc	–	5
BC-glyc-DCF1	0.5	5
BC-glyc-DCF2	1	5

[a]In respect to the volume of solution.

coated with evaporated carbon. 2 samples of each membrane were analyzed.

Tensile assays were performed on an Instron 5944 testing machine with Bluehill 3 software in tensile mode with a 1 kN load cell. The samples were strips of 70 mm × 5 mm and the gauge length 30 mm. At least 7 specimens were tested from each sample. The corresponding stress (MPa)–strain (%) curves were plotted, and the Young's modulus was determined from the slope of the low strain region in the vicinity of 0.05%.

BC and BC-diclofenac membranes were vacuum-dried during 24 h at room temperature before determining the swelling behavior. The samples were weighted, soaked in separate tubes containing 0.01 M phosphate buffer solution (pH 7.4) at room temperature (25 °C), and kept in this medium until a constant weight was attained. Before weighing the surface water of the wet membranes was gently removed with a tissue paper. The degree of swelling of the membranes (%) was calculated as: $[(W_{wet} - W_{dry})/W_{dry}] \times 100\%$ where W_{dry} and W_{wet} are the weights of dried and wet samples, respectively.

2.4. In vitro diclofenac release

2.4.1. Dissolution assays

BC-diclofenac dried membranes were immersed in a vessel containing 500 mL of a 0.01 M phosphate buffer (pH 7.4) solution. The dissolution was then carried out at 32 °C and 50 rpm. At determined time intervals, 5 mL of each solution was withdrawn, and the same volume of fresh buffer solution was added to maintain a constant volume. The diclofenac content in each aliquot was determined by UV–vis as described below. The diclofenac content at each time was plotted as a cumulative percentage release ($C_{cumulative} = C_n + (5 \times C_{n-1})/500$; where C_n and C_{n-1} are the diclofenac concentrations at time n and $n-1$). Six replicates were performed for each sample.

UV–vis quantitative analysis of diclofenac sodium salt was performed on a UV spectrophotometer (Evolution 600, Thermo Scientific) at 276 nm. A linear calibration curve ($y = 0.0376x - 0.0094$; $R^2 = 0.9995$) for diclofenac sodium salt in the range of 1–20 μg/mL was obtained at 276 nm.

2.4.2. Permeation assays

Human abdominal skin tissue from cosmetic surgery, obtained following informed consent, was used to produce epidermal membranes. Ethical approval was provided by the Ethics Committee of the Faculty of Health Sciences of the Lusófona University. After removal of the adipose tissue by blunt dissection, the epidermis was separated by immersing the skin in water at 60 °C for 1 min (Kligman & Christophel, 1963). It was then pinned on a cork-board, the epidermis was carefully peeled away from the dermis and mounted on filter paper, after which it was stored in a freezer at −20 °C until use. Prior to the diffusion experiments the epidermis was defrosted and cut to appropriate size. Permeation experiments ($n = 5$) with epidermal membranes were conducted on glass Franz type diffusion cells with a receptor volume of ~4 mL and a diffusional area of 0.95 cm^2. The continuously stirred receptor medium was isotonic phosphate buffered saline (PBS, pH 7.4). The receptor compartment was thermostated to 37 °C. A defined loading dose of diclofenac in different systems was placed in each donor compartment (Table 2). In the case of the solutions and gel, a micropipette was used for this purpose. BC membranes were cut to a size of 0.95 cm^2 that fitted the surface area of the donor compartment and covered the entire epidermal interface. At the start of the experiment 100 μL of PBS were applied on the BC membranes. The diffusion experiments were performed under occluded conditions by sealing the donor compartment with microscope coverslips. At designated time intervals the receiver solution was withdrawn completely from the receptor compartment and immediately replaced with fresh and pre-thermostated PBS. Quantitative analysis of the drug was performed by UV–vis as described above.

Table 2
Drug content and formulation amount applied in the permeation experiments with the different systems.

	Patch	BC-glyc-DCF1	Gel	BC-glyc-DCF2
% (w/w)	0.5	25	1	50
Dose (mg/cm^2)	1	1	2	2

One-way ANOVA with post Hoc tests (Tukey's HSD test) were used in this study (SPSS Statistics 17.0, IBM Corporation, Somers, NY, USA). A 0.05 significance level was adopted.

3. Results and discussion

3.1. Preparation and characterization of BC-diclofenac membranes

BC-diclofenac membranes with two distinct diclofenac contents, 1.0 and 2.0 mg/cm^2 (Table 2), were prepared by complete incorporation of an established volume of 0.5 and 1% diclofenac aqueous solutions into drained BC membranes, respectively. The objective of preparing BC-diclofenac membranes with this two distinct drug contents was to guarantee an accurate comparison with the commercial formulations used in the permeation essays. Glycerol was added to assure a good malleability of the membranes (BC-glyc-DCF; about 10 mg/cm^2 of glycerol) and improve drug absorption at higher concentrations. This simple and rapid methodology allowed us to determine accurately the mass of diclofenac incorporated into the BC membranes, which was additionally confirmed by weighting the dry BC-glyc-DCF membranes and by measuring, by UV–vis, the total amount of drug released in the dissolution assays.

BC-glyc-DCF dry membranes were very homogeneous (Fig. 2) indicating a good dispersion of diclofenac and glycerol inside the tridimensional nanofibrillar network of BC, without formation of aggregates.

The uniformity of the BC-glyc-DCF membranes was further confirmed by surface SEM analysis (Fig. 3). The SEM images disclosed also a good dispersion of diclofenac in the BC membranes surface,

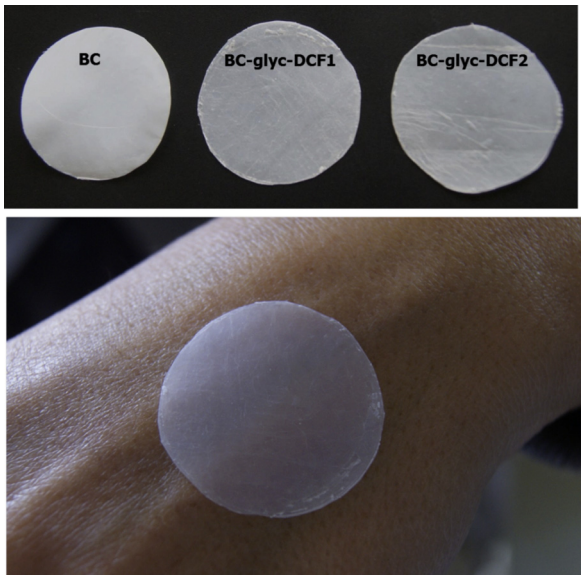

Fig. 2. Visual images of BC dry membranes (with 1.0 and 2.0 mg/cm^2), and one BC-glyc-DCF membranes with a good dermal adherence.

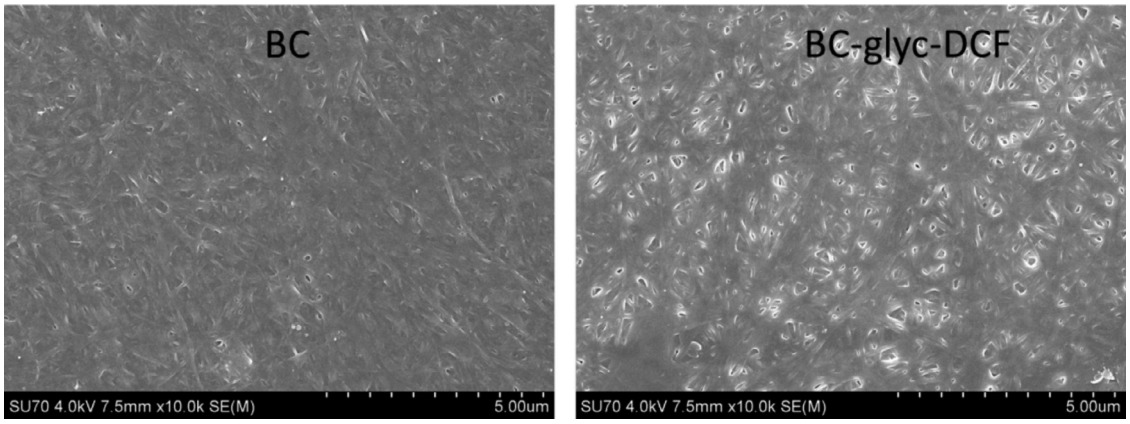

Fig. 3. SEM images of BC and BC-glyc-DCF.

Table 3
Young modulus, tensile strength and elongation at break of BC, BC-gly, BC-glyc-DCF1 and BC-glyc-DCF2 membranes, obtained from mechanical tensile assays. Mean values ± standard deviation, $n = 5$.

Sample	Young modulus (MPa)	Elongation at break (%)	Tensile strength (MPa)
BC	19132.32 ± 1286.15	2.26 ± 0.73	271.93 ± 62.06
BC-glyc	1128.22 ± 102.03	20.59 ± 1.96	90.24 ± 5.54
BC-glyc-DCF1	1554.45 ± 365.82	6.61 ± 1.00	52.66 ± 9.52
BC-glyc-DCF2	1333.34 ± 361.66	6.75 ± 1.35	49.80 ± 11.68

as no formation of considerable aggregates or crystallized drug is perceivable. In pure BC membranes fewer spaces between the nanofibrils were observed because of the collapse of the structure upon drying, while in the BC-glyc-DCF membranes these spaces were wider, showing a more open structure, because of the presence of glycerol and diclofenac. In particular, glycerol molecules will be retained inside the BC network, reducing the attraction forces between the nanofibrils, maintaining some mobility of the nanofibres and, therefore, avoiding their collapse during drying and keeping some flexibility.

In fact, the flexibility of the BC-gly-DCF membranes was very satisfactory guaranteeing an easier manipulation and a good adherence to the skin (Fig. 2). The increment of the flexibility of the BC-gly-DCF membranes was further confirmed by the increase of the elongation at break and the corresponding decrease on the Young moduli and tensile strength of the membranes, determined by tensile experiments (Table 3). However, the considerable decrease of Young moduli and tensile strength do not compromise the applicability of these materials in dermal drug release systems.

The structural characterization of the BC-glyc-DCF membranes was carried out by FTIR-ATR analysis. The spectra of BC, diclofenac, glycerol and BC-glyc-DCF are displayed in Fig. 4.

Pure BC membranes showed the typical FTIR spectrum of cellulosic materials, with the main absorption bands at around 3300, 2880, and 1100 cm^{-1}, attributed to the vibrations of the O—H, C—H and C—O—C groups, respectively. The FTIR spectrum of glycerol also presented a broad band associated with the O—H groups at 3250 cm^{-1} and the C—O vibrations typical of alcohols at 1030 and 1100 cm^{-1}. The spectrum of diclofenac displayed several typical absorption peaks, specifically at 3390 cm^{-1} (N—H stretching) 1573 cm^{-1} (COO— anti-symmetrical vibration), 1603 cm^{-1} (C=C ring skeletal vibration), 1350–1250 cm^{-1} (C—N stretching), and 730–745 (C—H out of plane, di- and tri-substituted rings). The BC-glyc-DCF spectrum is essentially a sum of the vibration peaks of all individual components, with no appearance of novel peaks or shifts in peaks position, indicating the nonattendance of complex interactions between BC, diclofenac and glycerol. Moreover, FTIR spectra collected at different points of the surface and inner layers of the diclofenac loaded membranes showed a similar profile, confirming the good dispersion of diclofenac (as well as glycerol) inside the membranes.

The swelling capacity of BC-glyc-DCF membranes (and of pure BC and BC-glycerol membranes for comparative purposes) is shown in Fig. 5.

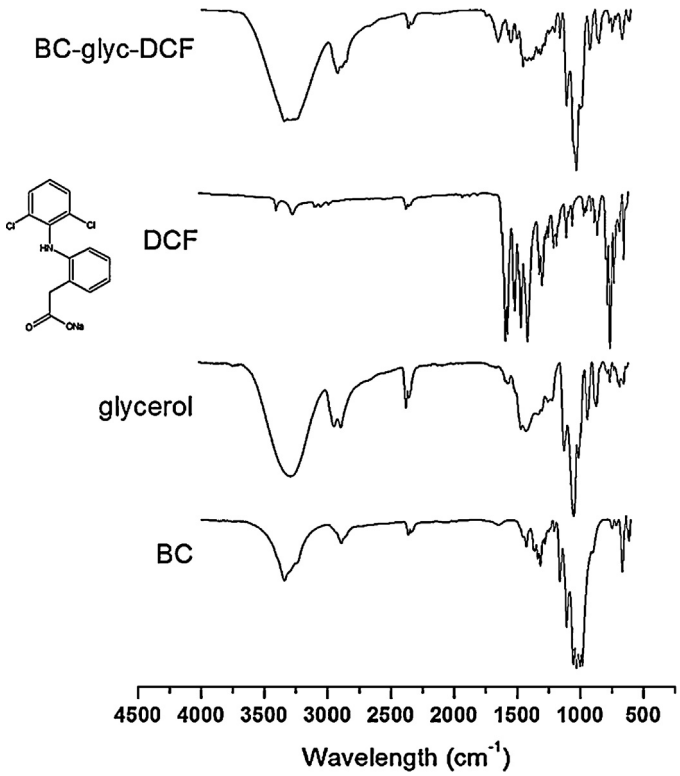

Fig. 4. FTIR-ATR spectra of BC membrane, glycerol, diclofenac sodium powder, and BC-glyc-DCF1 membrane.

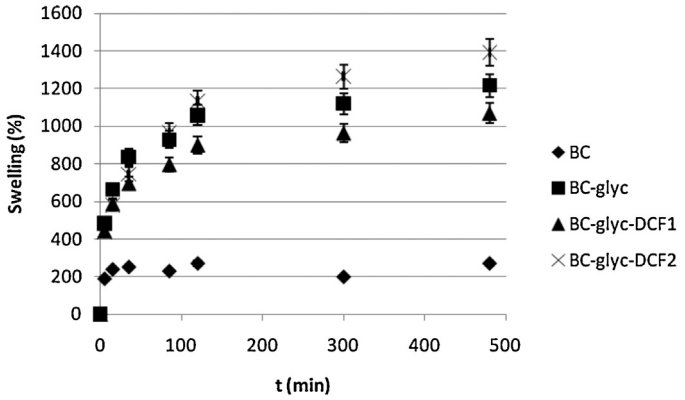

Fig. 5. Swelling behavior of BC, BC-glyc, BC-glyc-DCF1, and BC-glyc-DCF2 membranes. Mean values ± standard deviation, $n = 4$.

BC-glyc-DCF membranes presented an increased swelling ratio (up to 6 times higher) comparatively to pure BC membranes; because of the humectant and highly hydrophilic properties of glycerol which are known to have a strong influence in the swelling ability of the BC membrane (Trovatti et al., 2011). Moreover, as mentioned above, the presence of glycerol molecules limits the level of the collapse of the tridimensional structure of BC during drying, facilitating their re-hydration. In fact, BC-glycerol membranes (prepared with the same amount of glycerol) showed a very similar swelling behavior, indicating that diclofenac molecules had poor impact on this parameter. The good swelling behavior of the BC-glyc-DCF membranes is fundamental in the re-hydration in contact with skin, in the rate of release of diclofenac and in their ability to absorb exudates when applied to injured skin.

3.2. Dissolution assays

The release profile of diclofenac from BC-glyc-DCF membranes in a phosphate buffer solution is displayed in Fig. 6.

About 60% of the total drug was released in the first five minutes and more than 90% after 10 min. Since diclofenac is water soluble, its release from the BC membranes is essentially governed by its diffusion through the porous tridimensional network of BC. Consequently, under these conditions, the diffusion of the diclofenac molecules to the buffer solution is highly favored by the good swellability of the BC-glyc-DCF matrix, as formerly verified. Previous studies with BC-lidocaine membranes showed very similar dissolution profiles (Trovatti et al., 2011), however with a slower release rate (2 times lower). This difference indicates that the dissolution profile also depends on the chemical nature of the drugs and on their possible interactions established with the BC nanofibrils.

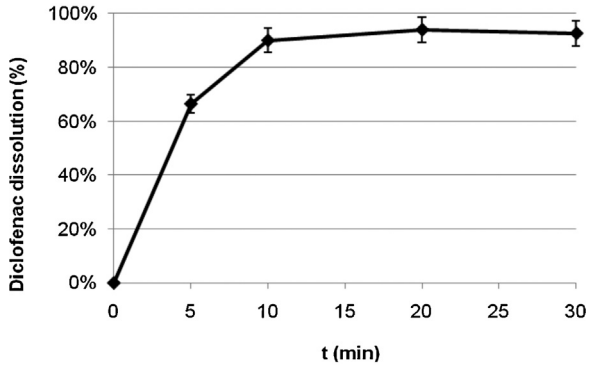

Fig. 6. Dissolution profile of BC-glyc-DCF1. Mean values ± standard deviation, $n = 3$.

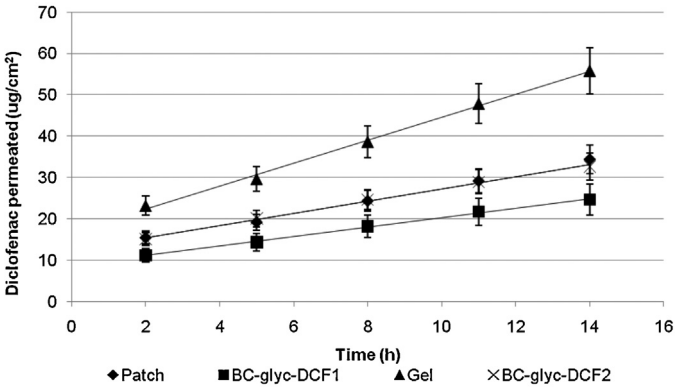

Fig. 7. Diclofenac sodium permeation across human epidermis. Mean values ± standard deviation, $n = 5$.

3.3. Permeation studies

The permeation profiles of diclofenac from BC-glyc-DCF membranes (BC-glyc-DCF1 and BC-glyc-DCF2) and two commercial formulations (patch and gel) through epidermal membranes are shown in Figs. 7 and 8.

Depending on the formulation system, different steady-state flux values (J_{ss}) were obtained; the highest diclofenac fluxes were attained with the commercial gel (3.24 μg cm^{-2} h^{-1}) and the lowest with the BC-gly-DCF1 (1.13 μg cm^{-2} h^{-1}) membrane. The commercial patch, the DCF-gly-DCF2 and the BC-gly-DCF1 membranes showed similar flux values, with no significant differences among them ($P < 0.05$). A comparison of the different diclofenac formulations based on the cumulative amount permeated after 10 h, as well as the permeability coefficient K_p, provided the same trends observed in the fluxes (Table 4).

Based on these results it can be assumed that the lower diclofenac fluxes obtained from BC membranes, compared with the gel, can be attributed to the resistance promoted by the complex tridimensional network of BC membranes to the diffusion of the drug molecules. In the commercial patch the drug is also entrapped in a matrix mounted on a polyester fibers scaffold, which explains the similarity of results in comparison to the BC membranes. It could be argued that there are differences in the doses applied with each system which could partly clarify the variations observed in fluxes. Nevertheless, the differences in the dose are very small and there are conflicting reports in the literature describing the effect of dose levels on drug permeation through skin (Brain, Walters, & Watkinson, 2002). These results are also in agreement with those

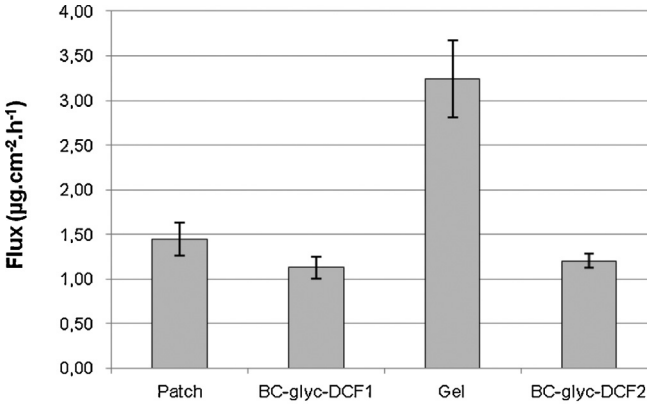

Fig. 8. Flux values for diclofenac sodium permeation from BC membranes with diclofenac sodium and comparative patch and gel. Mean values ± standard deviation, $n = 5$.

Table 4
Results for diclofenac percutaneous permeation. Mean values ± standard deviation, $n = 5$.

	J ($\mu g\,cm^{-2}\,h^{-1}$)	Cumulated DCF dose Q_{11h} (μg)	Diclofenac permeated in 11 h (% of the applied dose)	Permeability coefficient K_p ($cm\,h^{-1}$)
Patch	1.45 ± 0.19	29.13 ± 3.10	$2.91 \times 10^{-2} \pm 1.0 \times 10^{-3}$	$1.45 \times 10^{-3} \pm 1.9 \times 10^{-4}$
BC-glyc-DCF1	1.13 ± 0.12	23.60 ± 5.12	$2.36 \times 10^{-2} \pm 1.0 \times 10^{-3}$	$1.13 \times 10^{-3} \pm 1.2 \times 10^{-4}$
Gel	3.24 ± 0.43	40.81 ± 7.52	$2.04 \times 10^{-2} \pm 1.0 \times 10^{-3}$	$1.62 \times 10^{-4} \pm 2.2 \times 10^{-4}$
BC-glyc-DCF2	1.21 ± 0.08	27.85 ± 1.55	$1.39 \times 10^{-2} \pm 1.0 \times 10^{-3}$	$6.05 \times 10^{-4} \pm 4.0 \times 10^{-5}$

cited by Trovatti et al. (2011) in the studies comparing the performance of a lidocaine loaded BC membrane and a commercial gel.

Finally, similar release profiles have also been observed by using other diclofenac release systems. For instance, Liu et al. (2010) reported a slow release rate of diclofenac when encapsulated in solid lipid nanoparticles. Additionally, *in situ* nanosilica/acrylic acid grafted guargum membranes also provided excellent control over diclofenac release, which was attributed to high hydrophobicity and better drug retention by the well dispersed nanosilica particles (Giri et al., 2013).

Therefore, the current work confirms the capacity of BC membranes loaded with diclofenac to provide a sustained release, which can be successfully combined with its good biocompatibility and absorption properties.

4. Conclusion

BC-diclofenac membranes were prepared through a straightforward and very efficient approach. The drug loaded membranes were very homogeneous, considerably flexible and showed an increased swelling capacity (6 times higher) when compared to the pure BC membranes. The permeation rate of diclofenac in BC membranes was similar to that observed with the commercial patches and lower than that obtained with the gel, suggesting that this technology can be successfully applied to the transdermal delivery of diclofenac with the advantage of the easy application, simplicity of preparation and one single layer structure.

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