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Suitability of gamma irradiated chitosan based membranes as matrix in drug release system

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ABSTRACT

To test the possibility of obtain a material simultaneously biocompatible and microbiologically safe to be used as wound dressing material and as a matrix for drug release system, membranes with different initial contents in chitosan and 2-hydroxyethyl methacrylate (HEMA) have been prepared by gamma irradiation from a ^{60}Co source. The antimicrobial activity of obtained membranes against several reference strains was evaluated after inoculation. Sub-lethal gamma radiation doses were also applied in artificially contaminated membranes and the D_{values} of microorganisms in use were determined in order to predict which radiation dose could guarantee membranes microbiological safety. *In vitro* haemolysis tests were also performed using drug loaded membranes irradiated at different doses. Results point out that those membranes naturally exhibit antimicrobial properties. Also show that, over the studied range values, drug loaded irradiated membranes display a non-significant level of haemolysis. These features show that the application of prepared membranes as a transdermal drug release system "ready to use" is viable.

1. Introduction

Most drugs can be administered by a variety of routes, broadly defined as local and systemic. However, as the use of site-specific release systems can limit the adverse effects of systemic administration, it offers distinctive advantages over the classical methods of drug delivery. These include improvement of delivery efficacy, reduced toxicity and improved patient compliance and convenience (Sachdeva et al., 2010; Queiroz et al., 2001; Risbud et al., 2000).

The treatment of severe burn wounds that are easily infected is a serious problem. Infectious organisms preferentially target wounds beneath dressing materials, leading to serous infections that frequently require removal of the wound dressing and excision of cutaneous wounds. At the same time wound healing is a complex process involving various mechanisms, such as coagulation, inflammation, matrix synthesis and deposition, angiogenesis, fibroplasias, epithelization, contraction and remodelling (Alemdaroglu et al., 2006; Ishihara et al., 2002). Thus, the ideal wound dressing should protect the wound from bacterial infections but also provide a moist and healing environment and be biocompatible. In this way, the possibility of simultaneous rapid and proper healing of damage tissues, with local treatment of infections would be an important advantage.

Polysaccharides, like chitosan and its derivatives, have been considered to be advantageous in their application as a wound dressing material since they may, by themselves, actively participate in the process of wound healing (Alemdaroglu et al., 2006; Shi et al., 2006; Ishihara et al., 2002). In particular it is observed that chitosan oligosaccharides have a stimulatory effect on macrophages, and that they are chemo attractants for neutrophils *in vitro* and *in vivo*, an early event essential in accelerated wound healing (Enescu and Olteanu, 2008).

Chitosan is a linear polysaccharide composed of glucosamine and *N*-acetyl glucosamine units linked by β-(1-4) glycosidic bonds as shown in Fig. 1. Chitosan is obtained by *N*-deacetylation under alkaline conditions of chitin, the principal exoskeleton component in crustaceans and the second most abundant polysaccharide after cellulose in the amount of produced annually by biosynthesis (Alves and Mano, 2008; Singla and Chawla, 2001; Muzzarelli, 1973). Due to its unusual combination of intrinsic properties such as nontoxicity, biocompatibility, biodegradability, bioactivity, fat biding capacity, etc., biomedical applications of chitosan and its modified structures have been reported (Ngoenkam et al., 2010; Jayakumar et al., 2010; Alves and Mano, 2008; Torrado et al., 2004; Berger et al., 2004;

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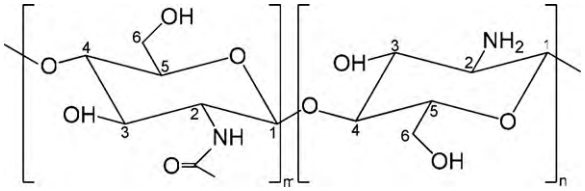

Fig. 1. Chemical structure of chitosan.

Thein-Han and Stevens, 2004; Khor and Lim, 2003; Madihally and Matthew, 1999; Koide, 1998). Most of the properties of chitosan can be related to its cationic nature. At acidic pH, it is a polyelectrolyte with a high charge density with one positive charge per glucosamine residue and, therefore, will interact with negatively charged molecules (Luyen and Huong, 1996).

Natural macromolecular materials such as chitosan are usually mechanically weak and often difficult to process as membranes. Among the various methods of modification used to improve chitosan properties and adequate it to the intend application, graft copolymerization has been the most used. Grafting of chitosan allows the formation of functional derivatives by covalent binding of a molecule onto the chitosan backbone. Chitosan bears two types of reactive groups that can be modified by grafting: the C-2 amino groups on deacetylated units and the hydroxyl groups on C-3 and C-6 carbons on acetylated or deacetylated units (Alves and Mano, 2008). In an acidic medium or without a catalyst, the reaction takes place at the amino group (Luyen and Huong, 1996).

2-Hydroxyethyl methacrylate is a synthetic polymer that when crosslinked can form a hydrogel that possesses high mechanical strength and biocompatibility (Bayramoglu and Arica, 2002).

With the aim of obtaining chitosan based membranes with dual effects, to accelerate wound healing due the bioactivities of chitosan itself and at same time be the supporting of a drug release system for prevention or treatment of bacterial infections, chitosan/pHEMA membranes were obtained by gamma irradiation from a ^{60}Co source (Fig. 2). The advantage of using gamma radiation in one final experimental/technological step is that it does not require the addition of chemical initiators or lethal agents to promote the polymerization reaction and, at the same time, promotes the inactivation of pathogenic microorganisms.

In previous work (Casimiro et al., 2005a,b, 2007) we have evaluated the effect of various synthesis conditions on some physical, chemical and microbiological properties, and presented drug release studies using amoxicillin, which is a moderate-spectrum antibiotic (active against a wide range of gram-positive and a limited range of gram-negative organisms) widely used for the treatment of skin infections among others infections. Here, the purpose of the study has been to assess the possibility of obtaining chitosan/pHEMA membranes simultaneously as a biocompatible and as a microbiologically safe dermal matrix for local drug release "ready to use". To evaluate the membranes features as potential biomaterial, microbiological and *in vitro* haemolysis tests were performed.

2. Materials and methods

2.1. Materials

Chitosan medium molecular weight (1.9×10^5 to 3.1×10^5 Da) 75–85% deacetylated was obtained from Aldrich Chemical Company, Inc., Milwaukee, USA. This was triturated ($\phi < 500\,\mu m$) and dried under vacuum at 40 °C. 2-Hydroxyethyl methacrylate (HEMA), stabilised, 98%, was obtained from ACROS Organics, Belgium, and used as received. All other chemical used were of analytical grade and used as received. Amoxicillin, minimum 97%, was obtained from Fluka, Germany.

2.2. Synthesis of chitosan/pHEMA membranes

The synthesis of chitosan/pHEMA membranes, here named as C*x*H*y*, where *x* indicates the percentage of chitosan (w/v) and *y* the percentage of HEMA (v/v) in the solution from which the membrane was obtained, was performed in a non-microbiological controlled environment at a common chemical laboratory.

The membranes synthesis was achieved by mixing different chitosan solutions (1 and 3% w/v of chitosan in acetic acid 1% v/v) with HEMA monomer (1 and 3% v/v of the final mixture). The bubble-free aqueous solutions (25, 40, and 55 ml) were poured on a clean glass plate ($\phi = 8$ cm) in a dust-free atmosphere and allowed to dry at room temperature. The membranes thus formed were washed with NaOH 1% and then with distilled water and carefully peeled off from the glass plate. Membranes were then blotted free of surface water with a filter paper, sealed in Amilon polyamide bags under nitrogen atmosphere and γ-irradiated at a dose rate (DR) of 0.6 kGy h^{-1} using a ^{60}Co gamma source with 295 kCi activity (November 2003), for several different intervals of time, at room temperature.

Samples were irradiated in a parallel position to the irradiator and amber and red perspex dosimeters (Harwell) were used to monitor the samples absorbed dose (with an error of ±2%).

All the samples studied presented a thickness value within 150 and 210 μm in the swollen form.

In the case of membranes drug loaded, previous to seal and irradiation procedures former described, membranes were dried to constant weight in vacuum at 40 °C and then immersed for 2 h in amoxicillin solution (100 mg/ml in saline solution (NaCl 0.9%, w/v)).

2.3. Microbiological strains

For the microbiological studies of chitosan/pHEMA membranes five potential pathogenic reference species were used. These included a gram-negative bacterium (*Escherichia coli* ATCC25922), three gram-positive bacteria (*Bacillus pumillus* ASSI MK1, *Staphylococcus aureus* ATCC 29213 and *Staphylococcus epidermidis* ATCC 12228) and one yeast (*Candida albicans* ATCC 10231).

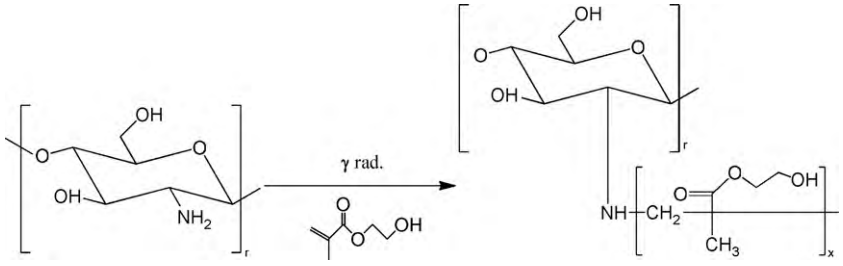

Fig. 2. Schematic representation of graft copolymerization of HEMA onto chitosan.

2.4. Characterisation of chitosan/pHEMA membranes

2.4.1. Microbiological studies

2.4.1.1. Bioburden characterisation. In previous work the number of contaminating microorganisms that naturally occur on non-irradiated membranes with different initial contents in chitosan and HEMA was determined (Casimiro et al., 2005b). Here, the different types of colonies obtained in the bioburden determination were morphologically and biochemically characterised using microscopy, gram staining and catalase and oxidase tests.

2.4.1.2. Natural antimicrobial activity evaluation. To evaluate the natural antimicrobial activity of the obtained membranes, non-irradiated membranes were used to assure that the observed microorganisms inactivation was due to the chitosan presence and not to the lethal characteristics of γ radiation as sterilization agent.

Membranes with different initial contents in chitosan (C1H1 and C3H1) were artificially contaminated with the reference strains of *E. coli*, *Bacillus subtilis*, *S. epidermidis* and *C. albicans*.

The strains were cultured overnight at 37 °C on Tryptic Soy Agar (TSA), Oxoid, UK. The microorganisms were then suspended in a sterile saline solution and as result suspensions of 10^8 cells/ml were obtained for each strain. Afterwards, for each strain in use, each membrane was inoculated with 0.1 ml of the obtained suspension and put in sterile covered Petri plate for 2.5, 16 and 24 h at room temperature. To calculate the number of survivors after the period contact, membranes were put in 100 ml of sterile saline solution for 15 min with magnetic stirring. Consecutive dilute solutions were prepared by taking 1 ml from previous solution and mixed with 9 ml of sterile saline solution. Six aliquots were plated in TSA medium and incubated at 30 °C for *B. subtilis* and *C. albicans*, and incubated at 37 °C for *E. coli* and *S. epidermidis*. Colony forming units per membrane (cfu/membrane) were counted during 1 week. As control 0.1 ml of each suspension strain was put in an empty sterile covered Petri plate for the same periods of time in order to evaluate the decrease of the microbiological population when not in contact with the membranes.

2.4.1.3. Gamma radiation inactivation studies of reference strains. As the obtained membranes were naturally low contaminated (346 ± 48 and 22 ± 26 cfu/membrane (mean ± SD) for C1H1 and C3H1, respectively) (Casimiro et al., 2005b) membranes of lower chitosan content (which were the one expected to exhibit low antimicrobial activity) were artificially contaminated with reference strains of *E. coli*, *B. subtilis*, *S. aureus* and *C. albicans* mentioned before. To evaluate the inactivation response of each reference strain, these artificially contaminated membranes were then exposed to sub-lethal gamma radiation doses and D_{10} values were calculated based on each reference strain survival curve. The D_{10} value represents the necessary dose to inactivated 90% of the initial microbiological population.

A previously validated method was used: each membrane was placed in a sterile bag and contaminated with a microorganism saline solution using *ca.* 10^7 to 10^8 cfu/membrane. The bags were sealed and gamma irradiated with a range of irradiation doses of 0.5 up to 4 kGy for all the studied strains. To calculate the number of survivors, and the initial contamination (at 0 kGy), each membrane was then washed in 100 ml of sterile saline solution and stirred by a stomacher (Stomacher 3500, Seward, UK) for 15 min. Six aliquots were plated in TSA medium and incubated at 30 °C for *B. subtilis* and *C. albicans*, and at 37 °C for *E. coli* and *S. aureus*. Colony forming units per membrane were counted for 1 week.

2.4.2. Haemocompatibility studies

Biological characterisation of the obtained membranes involved *in vitro* haemolysis assays which were performed according to the standards of International Standard Organization 10993-4 (ISO, 1999) and American Society Testing and Materials 756-00 (ASTM, 2000). These assays evaluate the toxic effect of artificial materials on the blood cells *in vitro*.

Since one of the objectives of the current work is to obtain a microbiologically safe matrix for local drug release, the blood compatibility studies have focused only γ-irradiated amoxicillin loaded membranes, in particular C1H1 type membranes. The selection of radiation doses values of 5 and 10 kGy instead of the standard sterilization dose of 25 kGy was done based on membranes bioburden and radiation resistance of (inoculated) microorganisms. Moreover, and in spite of other studies have reported the feasibility of gamma radiation (25 kGy) as a sterilization method of amoxicillin (Valvo et al., 1999), lower radiation doses would minimize eventual induced degradation products as well as adverse side effects on physical and chemical matrix properties (Casimiro et al., 2005a; Lim et al., 1998).

Rabbit venous blood was collected in polypropylene tubes containing anticoagulant acid citrate solution (ACD) at a citrate/blood ratio of 1:9. This blood was then diluted with physiological buffer saline solution (PBS) at the concentration of 1.27 ± 0.01 mg/ml. 5.6 ml of the obtained solution were incubated with pieces of membranes at 37 °C for 3 h without agitation. After the contact period the suspension was centrifuged at 700–800 × *g* and haemoglobin concentration ([Hb]) was determined on the supernatant. The Hb content was determined with a spectrophotometric method which is based on the conversion of haemoglobin (by reaction with potassium ferricyanide and potassium cyanide) into cyanomethaemoglobin (that exhibits a peak at 540 nm). As negative control a dilute blood solution (the same mentioned before), and as positive control, an identical concentration blood solution but water diluted instead of PBS diluted were used. The percentage of haemolysis was calculated as described as follows:

$$\text{Haemolysis } (\%) = \frac{[\text{Hb}]_{\text{sample}} - [\text{Hb}]_{\text{negative control}}}{[\text{Hb}]_{\text{positive control}} - [\text{Hb}]_{\text{negative control}}} \times 100 \quad (1)$$

3. Results and discussion

3.1. Microbiological studies

3.1.1. Bioburden characterisation

In accordance with the literature (ISO, 1995; EN, 1994), the determination and characterisation of contaminating microorganisms that naturally occur on the obtained membranes are a first approach to predict the dose needed to achieve a predetermined target of sterility.

The bioburden characterisation showed that all microorganisms are gram-positive and catalase-positive. However, depending on chitosan membrane content it is possible to identify two distinct morphological types. The overall microbiota existent on C1H*y* type membranes are gram and catalase-positive, and oxidase-negative cocci, while the microorganisms existent on membranes with higher chitosan content (C3H*y* type membranes), independently of HEMA concentration, are gram-positive, catalase-positive and oxidase-positive cocci. Moreover, if HEMA content is the one being considered, it can be seen that membranes with higher HEMA (C*x*H3) concentrations present nonmotile rods while C*x*H1 membranes show motile rods.

3.1.2. Natural antimicrobial activity evaluation

To evaluate if obtained membranes preserve the natural antimicrobiological activity of chitosan four reference strains were tested.

Table 1 shows the logarithmical inactivation of the tested strains (based on the strain survival values) due to different membrane contact periods and different membrane content in chitosan. The

Table 1
Antimicrobial activity of membranes—logarithmical inactivation of the tested strains due to different membrane contact periods and different membrane content in chitosan (mean ± SD; $n = 6$).

Membrane	Incubation time	Antimicrobial activity (log cfu/membrane)							
		E. coli		*B. subtilis*		*S. epidermidis*		*C. albicans*	
		Control	Matrix	Control	Matrix	Control	Matrix	Control	Matrix
C1H1	2.5 h	7.71 ± 0.16	6.75 ± 0.31	7.54 ± 0.09	6.60 ± 0.12	7.84 ± 0.08	6.99 ± 0.16	7.03 ± 0.08	6.21 ± 0.24
	16 h	7.47 ± 0.11	6.43 ± 0.22	6.75 ± 0.07	5.98 ± 0.09	7.64 ± 0.03	6.68 ± 0.06	7.22 ± 0.12	6.23 ± 0.20
	24 h	6.99 ± 0.40	6.87 ± 0.09	6.88 ± 0.61	5.67 ± 0.07	7.38 ± 0.53	6.31 ± 0.12	7.10 ± 0.03	6.23 ± 0.14
C3H1	2.5 h	7.71 ± 0.16	6.95 ± 0.31	7.54 ± 0.08	6.64 ± 0.06	7.84 ± 0.08	6.72 ± 0.06	7.03 ± 0.08	6.06 ± 0.12
	16 h	7.47 ± 0.11	6.20 ± 0.22	6.75 ± 0.07	5.55 ± 0.20	7.64 ± 0.03	6.37 ± 0.14	7.22 ± 0.12	6.07 ± 0.09
	24 h	6.99 ± 0.40	6.24 ± 0.16	6.88 ± 0.61	5.55 ± 0.26	7.38 ± 0.53	5.76 ± 0.20	7.10 ± 0.03	6.40 ± 0.10

Table 2
Inactivation equations and required dose to inactivated 90% of the (inoculated) microbial population (D_{10}) in C1H1 type chitosan/pHEMA membranes.

Artificial contamination (cfu/membrane)	Reference strains	$\log N = kD + \log N_0$	r^2	D_{10} ± SD (kGy)
10^7 to 10^8	*Escherichia coli*	n.d.[a]	–	–
	Staphylococcus aureus	$\log N = -5.11D + 6.95$	0.999	0.20 ± 0.01
	Candida albicans	$\log N = -2.05D + 5.27$	0.989	0.49 ± 0.05
	Bacillus subtilis	$\log N = -0.96D + 4.88$	0.837	1.04 ± 0.98

[a] n.d. – not determined.

obtained data point out to an inhibition of the growth of all microorganisms tested for the different contact periods with the non-irradiated chitosan/pHEMA membranes.

However, in contrast with the response of *B. subtilis* and *S. epidermidis* (gram-positive bacteria), that show an increasing inactivation with membrane time contact and membrane content in chitosan, the inactivation of *E. coli* and *C. albicans* (gram-negative and yeast, respectively) does not seem to be linearly dependent on chitosan content or membrane time contact.

Chitosan has been described as a material being able of inhibit microbial growth due to the interaction of its cationic amino groups with the microorganism anionic cell surface. This process results in changes of permeability that causes the disruption of mass transport across the cell and consequently the decrease of the viable cell number (Muzzarelli et al., 1990).

As gram-positive and gram-negative microorganisms differ from cell wall structure, it is expected that they exhibit different responses to chitosan interaction as observed and as has been reported (No et al., 2002; Helander et al., 2001).

3.1.3. Gamma radiation inactivation studies of reference strains

In order to predict which radiation dose could assure membranes microbiological safety, *i.e.*, to predict the dose needed to achieve a predetermined target of sterility or sterility assurance level (SAL), sub-lethal gamma radiation doses were applied in the artificially contaminated membranes of lower chitosan content (C1H1). After γ irradiation with several doses the D_{10} of each reference strain was calculated based on survival curves. The correlation between the absorbed dose D (kGy) and the microorganisms' survival number N (cfu/membrane) is represented by:

$$\log N = kD + \log N_0 \quad (2)$$

in which N_0 is the initial microbiological population. The D_{10} value is given by the inverse slope $(-1/k)$ of the obtained linear curve.

The inactivation equations and D_{values} are listed in Table 2. Results are in agreement with data reported at literature (Whitby, 1993; Silverman, 1983), which mention *E. coli* as a microorganism of high sensibility to γ radiation, reason why there is no viable cells at 0.5 kGy, and *Bacillus* spp. as one of the radiation resistant microorganisms.

Considering the application of a dose of 4–5 kGy and based on the obtained results (knowing that 90% of inactivation represents 1 log decrease on cfu counting), it is expect a population reduction of 25 logarithms for *S. aureus*, 10 logarithms for *C. albicans* and 5 logarithms for *B. subtilis* at a bioburden level of 10^7 to 10^8 cfu/membrane. Thus, a dose of 5 kGy would be sufficient to obtain a SAL of 10^{-5} (1 item contaminated in 100,000 items). However, as the number of contaminating microorganisms that naturally occur on non-irradiated membranes ($\approx 3 \times 10^2$ cfu/membrane) is much lower than the number present on inoculated ones (10^7 to 10^8 cfu/membrane), it is possible to assure that the exposure to 4–5 kGy allows obtaining membranes microbiologically safe (SAL would be $\ll 10^{-5}$). Nevertheless, it would be necessary to apply specific microbiological methodology in order to validate the sterilization procedure, reason why it is used the term "microbiological safe" instead of sterile.

3.2. Haemolysis tests

The *in vitro* haemolysis test performed allowed to quantify the haemoglobin released by red blood cells haemolysis due to the contact with C1H1 irradiated drug loaded membranes. The obtained values are presented in Fig. 3 (data represents mean ± SD from three independent experiments).

Haemolysis is regarded as an especially significant screening test, since it provides quantification of small levels of plasma haemoglobin which may not be measurable under *in vivo* conditions. According to American Society Testing and Materials (ASTM,

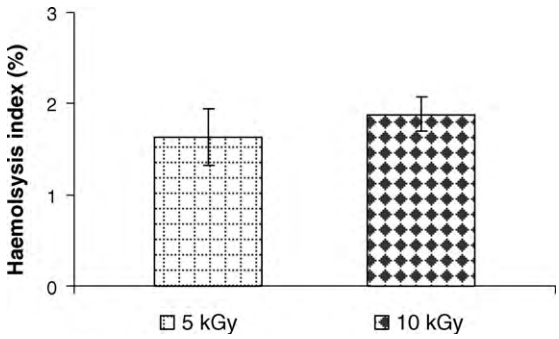

Fig. 3. Values of haemolysis index (%) induced by irradiated drug immobilized C1H1 chitosan/pHEMA membranes (DR = 0.6 kGy h^{-1}). Data are expressed as mean ± SD ($n = 3$).

2000) materials can be classified in three different categories according to their haemolytic index (haemolysis %). Materials with percentages of haemolysis bellow 2% are considered non-haemolytic material, the ones with haemolytic index between 2 and 5% are classified as slightly haemolytic, and finally when the material presents a haemolysis percentage over 5% is considered a haemolytic material. Although by definition a blood-compatible material should be non-haemolytic, in practise several medical devices cause haemolysis. This means that when haemolytic effect takes place, it is important to make sure that clinical benefits overcome the risks and that the values of haemolysis are within acceptable limits.

In the present case, results point out a non-significant level of *in vitro* haemolytic activity since both results, at 5 kGy and at 10 kGy, present a haemolysis degree lower than 2%. These results evidence the haemocompatible character of the obtained drug loaded membranes, which shows potential biomedical application.

4. Conclusions

The goal of the present study is to obtain, by gamma irradiation, biocompatible and microbiological safe chitosan/pHEMA membranes suitable to deliver transdermal antibiotics and already "ready to use". Obtained results show not only that due to intrinsic antimicrobial properties of obtained chitosan/pHEMA membranes, low radiation doses (5 kGy) are enough to assure microbiological safe membranes, but also that irradiated membranes (drug loaded) display an insignificant level of haemolysis which shows potential biomedical application. Moreover, the present study also evidences one of the advantages of the radiation technique by allow combining the synthesis/modification and microbiological safety in a single γ irradiation experimental step. The readiness, simplicity and reproducibility of these membranes open good perspectives to its use in clinical media. However, additional *in vivo* studies are required to achieve that goal.

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