# Macroporous poly(ϵ-caprolactone) with antimicrobial activity obtained by iodine polymerization

**Kleber de Arruda Almeida,[1] Alvaro Antonio Alencar de Queiroz,[1] Olga Zazuco Higa,[2] Gustavo Abel Abraham,[3] Julio San Román[3]**

[1]*Departamento de Física e Química/Instituto de Ciências, Universidade Federal de Itajubá (UNIFEI), Av. BPS. 1303, 37500-903, Itajubá, Minas Gerais, Brasil*

[2]*Laboratório de Biologia Molecular, Instituto de Pesquisas Energéticas e Nucleares-IPEN/CNEN-SP. Av. Prof. Lineu Prestes 2242, Cidade Universitária, 05508-000, São Paulo-SP/Brasil*

[3]*Instituto de Ciencia y Tecnología de Polímeros, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain*

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**Abstract:** The most serious problem usually encountered in the field of implanted biomedical devices is infectious morbidity as a primary source of mortality. In this work, the synthesis and characterization of a macroporous iodine-based sanitizer (iodophor), poly(caprolactone)-iodine (PCL-$I_2$), are presented. Characterization methods include nuclear magnetic resonance spectroscopy, gel permeation chromatography, nitrogen adsorption-desorption, and scanning electron microscopy. The *in vitro* cytotoxicity to CHO cells based on cell viability with Chinese hamster ovary cells (CHO) and antimicrobial activities against *Escherichia coli* and *Staphylococcus aureus* were examined. The obtained macropore PCL-$I_2$ structures had a rather narrow size distribution. The PCL-$I_2$ iodophor was noncytotoxic to Chinese hamster ovary cells. The antimicrobial activities of the PCL-$I_2$ were assessed against *E. coli* and *S. aureus.* The tested PCL-$I_2$ showed better antimicrobial activity against *E. coli* than against *S. aureus*. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 68A: 473–478, 2004

**Key words:** antimicrobial activity; poly(ϵ-caprolactone); biological activity; iodophor

## INTRODUCTION

It is well known that one of the most serious problems usually encountered in the field of implanted biomedical devices is infectious morbidity as a primary source of mortality.[1–3] The attachment of bacteria to solid biomaterial surfaces is an important phenomenon because of its possible role as the very first step in the development of an infection.

The usefulness of systemic administration of antibiotics to the infection treatment after biomaterials implantation depends mainly on the absorption kinetics and the spectrum action of the drug, reinforcing the consensus that topical therapy has a very important role in the restriction of the infection area, thus reducing the risks of septicemia.[4,5]

Since the iodophor poly(vinylpyrrolidone)-iodine (povidone-iodine) was reported to have antimicrobial properties in the 1960s, extensive studies have reinforced their antibacterial, antifungal, and antivirus properties.[6–16] However, the high toxicity of povidone-iodine at higher iodine concentrations, their water-solubility, limited viability of the iodine at low concentrations, and the risks of immunological rejection, limit the use of the iodophor in treatment of superficial burn injuries.[17–19]

Poly(ϵ-caprolactone) (PCL), an aliphatic polyester often used in a number of medical and drug delivery devices with United States Food and Drug Administration approval, has been used as wound-covering material and matrix for tissue engineering.[20–22]

Recently, we have reported that iodine promotes ring-opening polymerization of ϵ-caprolactone (ϵ-CL) under mild conditions, with a moderate rate, high degree of conversion, high molecular weight, and good stereoregularity.[23]

The bulk polymerization promoted by iodine induces a phase inversion in the reaction medium, producing the charge transfer complex PCL-iodine (PCL-I). As a consequence of the phase inversion, the synthesis of PCL-I structures with both higher degree of porosity and available/free iodine concentrations may be attained.

To the best of our knowledge, the macroporous iodophor PCL-I has not been reported in the literature

*Correspondence to:* A. A. A. de Queiroz; e-mail: alencar@efei.br

and this appears to be the first study of its antimicrobial activity. The present study was undertaken to determine the *in vitro* antimicrobial activity of PCL-I against *Escherichia coli* and *Staphylococcus aureus*, the potential infecting microorganisms in the surgical implant area.

## MATERIALS AND METHODS

### Synthesis and physicochemical characterization

The monomer ϵ-CL, obtained from Aldrich, was dried with $CaH_2$ for 24 h and then distilled under reduced pressure. Iodine-induced ϵ-CL polymerization was performed in bulk using septum-stoppered flasks in a bath, which was protected from light. The reaction temperature was controlled by an immersion bath and maintained at 25°C. Stoichiometric amounts of purified and pulverized iodine (Merck) were added directly with vigorous agitation into ϵ-CL under nitrogen flow, the monomer-initiator ratio being $[\epsilon\text{-CL}]/[I_2] = 20{:}10$. Reaction time was taken to start at the moment when the iodine was added. Afterward, the reaction mixture was precipitated with a large excess of cold ethanol. Total available iodine was assayed in PCL-I by potentiometric titration with standardized sodium thiosulfate.

The maximal level of free iodine concentration in PCL-I was maintained at 10% of available iodine to get the maximal antimicrobial activity and to avoid possible effects of irritant contact dermatitis.

The obtained polyester was characterized by different techniques. The reaction conversion and chemical structure of the polyester was evaluated by proton nuclear magnetic resonance ($^1$H NMR) spectroscopy. Spectra were performed in a Varian UNITY-400 NMR spectrometer. Chemical shifts in parts per million (ppm) were reported downfield from 0.00 ppm using tetramethylsilane as the internal reference.

Molecular weights and molecular weight distributions were determined by gel permeation chromatography (GPC) using a PerkinElmer gel permeation chromatograph equipped with a refractive index detector series 200. A set of 104, 103, and 500 Å PL-gel columns conditioned at 25°C were used to elude the samples of 10 mg/mL concentration at 1 mL/min high-performance liquid chromatography-grade chloroform flow rate. The weight-average ($M_w$) and number-average ($M_n$) molecular weights were determined using a calibration curve with polystyrene standards. The polydispersity index was calculated as the $M_w/M_n$ ratio.

Surface areas of PCL-I were determined from conventional $N_2$ sorption isotherms utilizing the BET theory.[24,25] The Dubinin-Radushkevich equation and the density functional theory formalisms were used to calculate the pore volume, from which the pore surface area and pore size distribution were determined.[26] The samples (0.3–0.8 g) were previously degassed at 298 K during approximately 24 h and then studied at 77 K.

The pore morphology of PCL-I samples was observed by scanning electron microscopy (SEM) using a Phillips JEOL XL 30 microscope.

### Antimicrobial activity

The *in vitro* antimicrobial activities against *E. coli* and *S. aureus* (ATCC 6538 P) were determined on the powdery PCL-I samples by the cut plug method on nutrient agar which contained peptone ($10\ g \cdot L^{-1}$), NaCl ($5\ g \cdot L^{-1}$), beef extract ($5\ g \cdot L^{-1}$), and agar ($20\ g \cdot L^{-1}$) at physiological pH.[27–29] The assay plates were seeded with the test bacteria and, after solidification, the wells were made and filled with 20 mg of the powdery polymer. The plates were incubated at 30°C for 24 h after which the diameters of inhibition zones were measured. The compounds produced inhibition zones that were further assayed at different concentrations in aqueous suspension in order to quantify their inhibitory effects. A loopful of each culture was placed into test tubes containing 10.0 mL of 10-fold diluted sterile nutrient broth, and then incubated overnight at 30°C. At this stage, the cultures of the test bacteria containing $6 \times 10^4$ cells/mL were used for the antimicrobial test.

Because the PCL-I is not water soluble, it was suspended in the dilute of the above nutrient broth medium to make $0.05\ g \cdot mL^{-1}$ and 0.5 mL was transferred to flasks containing sterile 10-fold diluted nutrient to give the final concentrations of 10.5 and 2.5 $mg \cdot mL^{-1}$. Exposure of the bacterial cells was started when 0.2 mL of the culture containing $6 \times 10^4$ bacterial cells $\cdot\ mL^{-1}$ was added to 10 mL of the above PCL-I suspension and shaken at 30°C. At the same time, 0.2 mL of the same culture was added to 10 mL of the nutrient broth solution, decimal solutions were prepared, and the starting number of cells was counted by spread plate method. After 24 h contact, 1.0-mL portions were removed and mixed with 9.0 mL of 10-fold diluted nutrient broth and then decimal serial solutions were prepared from these dilutions.

The surviving bacteria were counted by the spread plate method. After inoculation, the plates were inoculated at 30°C and the number of colonies was counted after 24 h. The ratio was performed in triplicate every time. The ratio of the colony numbers for the media containing the PCL-I and those without the polymer was taken as surviving cell number. Finally, the antimicrobial activity was evaluated from this value.

### Cytotoxicity evaluation

The cytotoxicity of PCL-I extracts was evaluated against Chinese hamster ovary (CHO) cells, ATCC CHO k1 [American Type Culture Collection (ATCC)], according to ISO guidelines.[30,31]

Serially diluted PCL-I extracts were added to a CHO cell culture. CHO cells were grown in Eagle minimum essential medium (MEM)–fetal calf serum (FCS) and 1% penicillin-streptomycin solution, in a plastic tissue culture flask, at 37°C/5% $CO_2$. After confluent monolayer propagation, the culture medium was removed and the cells were washed with calcium- and magnesium-free phosphate saline buffer. The culture was treated with 0.25% trypsin solution to detach the cells from the culture tissue flask. After trypsinization, the cells were transferred to a screw-capped plastic tube, centrifuged, and washed twice with calcium- and magnesium-free phosphate saline

buffer. The cells were resuspended in MEM-FCS and adjusted to give $1 \times 10^2$ cells/mL. A volume of 2 mL of this cell suspension was seeded to each 60-mm-diameter assay culture dish and incubated for about 5 h for adhesion of the cells. The culture medium was then replaced by 5 mL of fresh MEM-FCS, in the control plates, and by undiluted (100%) and successively diluted extracts (50, 25, 12.5, and 6.25%), in culture dishes with the adhered cells. All concentrations were tested in triplicate. After incubation of the culture dishes for 7 days at 37°C, 5% $CO_2$ for the cell colonies formation, the PCL-I cytotoxicity was evaluated quantitatively, based on cell viability.

After the incubation time, the medium was removed from the dishes and after fixation with buffered saline formalin solution (10% formaldehyde), the colonies were stained with Giemsa. The number of visible colonies on each dish was counted and compared with the number of colonies in the CHO control dish. Phenol solution (0.02%) and PCL extract (60 $cm^2$ in 60 mL MEM-FCS) were used as positive and negative controls, respectively. The results were expressed as percent cell survival from control per volume of extract tested.

## RESULTS AND DISCUSSION

$^1$H NMR spectrum of PCL-I obtained after 2.5 h reaction time is shown in Figure 1. The assignments of the chemical shifts of PCL obtained by iodine-promoted polymerization are in excellent agreement to those reported in the literature.[32] The presence of signals associated to the residual monomer allowed the calculation of the monomer conversion. It was previously reported that higher levels of monomer conversion may be achieved by increasing the [ϵ-CL]/[$I_2$] molar ratio.[23]

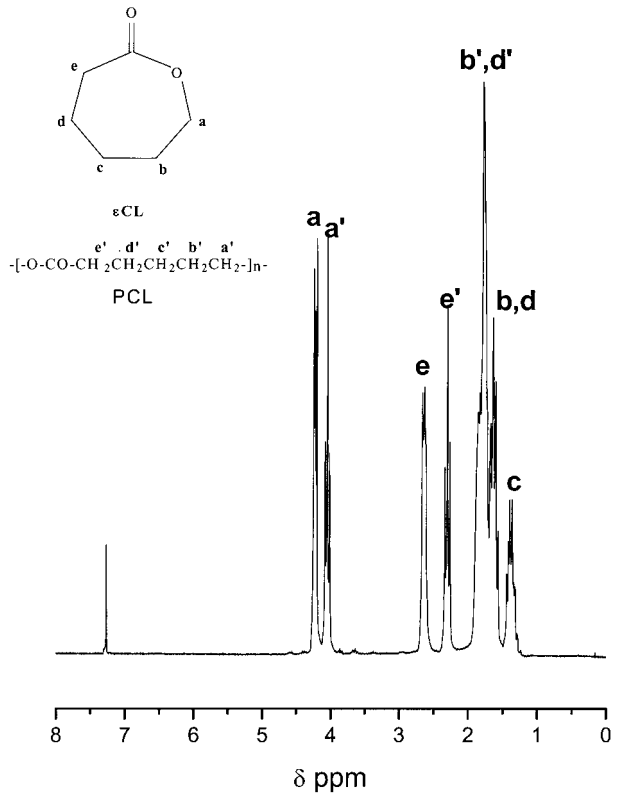


**Figure 1.** $^1$H NMR spectra recorded in $CDCl_3$ of the reaction mixture at low conversion (2.5 h reaction time).

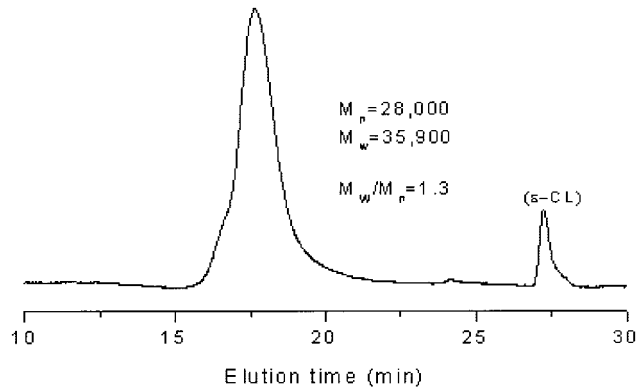


**Figure 2.** GPC profile of iodine-promoted polymerization of ϵ-CL.

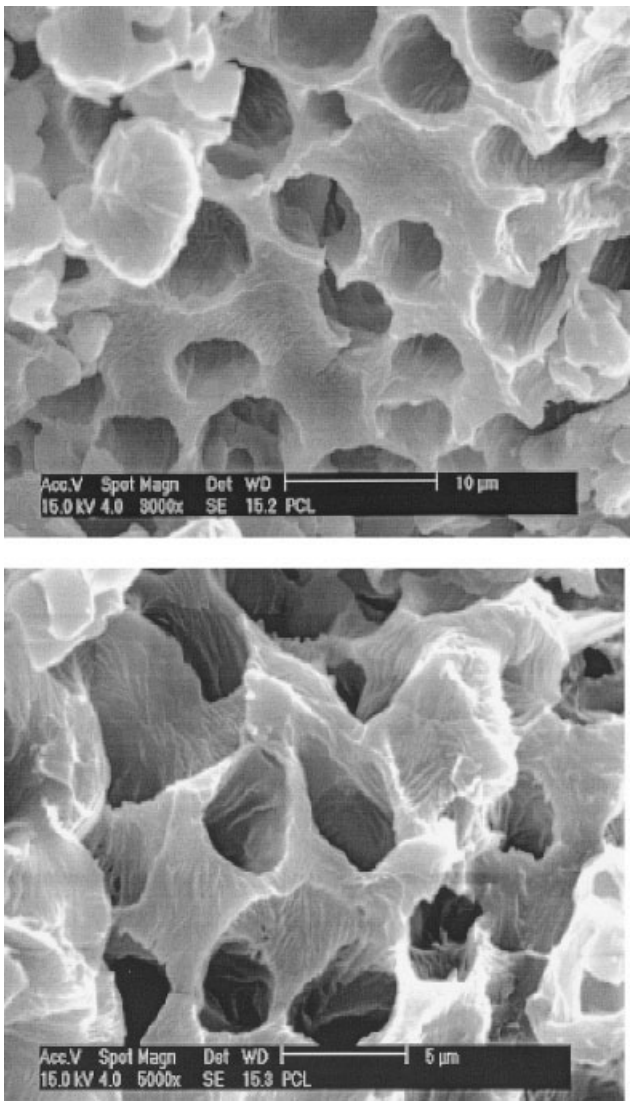


**Figure 3.** SEM micrographs of the PCL-I particles obtained by the iodine bulk polymerization.

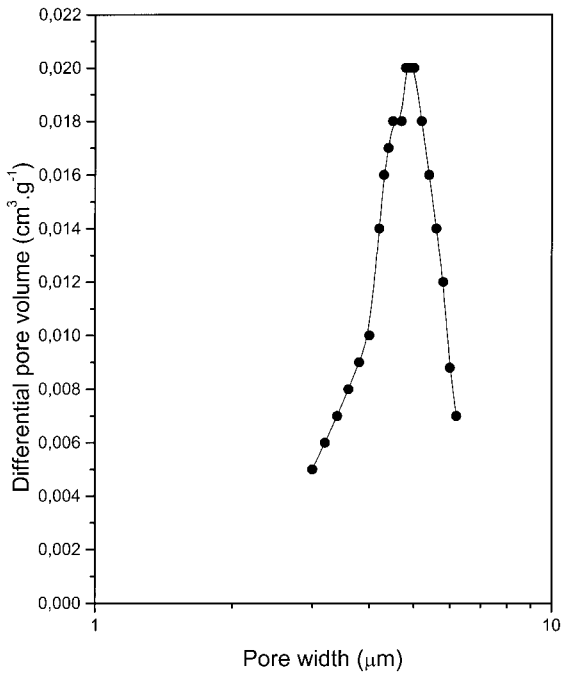


**Figure 4.** Differential pore size distributions for PCL-I calculated by application of the density functional theory method to $N_2$ adsorption isotherms at 77 K.

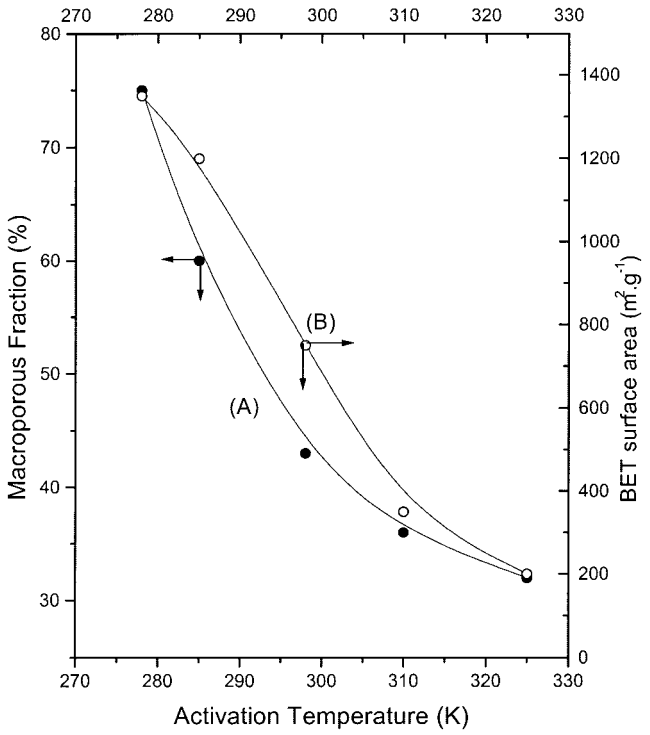


**Figure 5.** Effects of activation temperature on the macropore fraction (A) and BET surface area (B).

Figure 2 illustrates the GPC traces for PCL-I obtained by bulk iodine polymerization at 25°C. The molecular weight distribution is very narrow, with a polydispersity value ($M_w/M_n$) of 1.3 and an apparent molecular weight ($M_w$) of 35.900.

SEM micrographs (Fig. 3) of the synthesized PCL-I displayed particles with pore diameter larger than 2 μm, forming an interconnected sponge-like structure. According to the definition of IUPAC,[33] porous materials can be divided into three types according to their pore diameters: microporous (<2 nm), mesoporous (2–50 nm), and macroporous (>50 nm). Based on this terminology, PCL-I structure has a macroporous structure. The macropores observed in the particles were closely circular and they had rather uniform sizes.

The size distribution of pores calculated from the $N_2$ adsorption isotherms is shown in Figure 4. PCL-I exhibits a monodisperse distribution, with pores comprised between 3 and 5 μm. Because of the porous sponge-like structure of the PCL-I, an improvement on the desirable properties of a wound dressing such as high permeability to oxygen and better water uptaking ability relative to the nonporous PCL may be expected. The maintenance of the monodisperse porous structures may be desirable to the development of systems with controlled small-to-moderate molecule release and high absorption of wound exudates providing a moist environment and three-dimensional structure for cell growth and tissue respiration.

The macropore fraction of PCL-I plotted against activation temperature of the reaction medium is shown in Figure 5. The macropore fraction decreased continually with the increase of activation temperature, indicating coalescence of the pore structures in

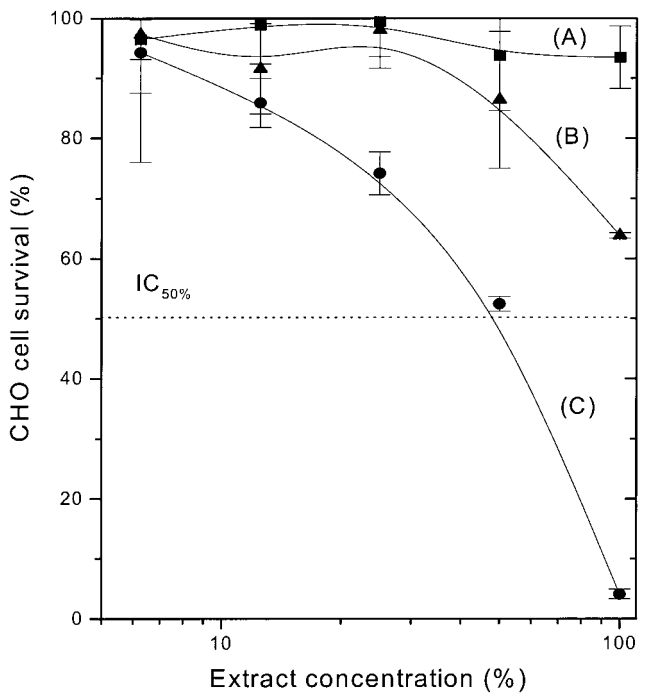


**Figure 6.** Cytotoxicity profile of PCL-$I_2$ (B) extracts against CHO cells; negative control (high-density polyethylene) (A); and positive control (phenol) (C).

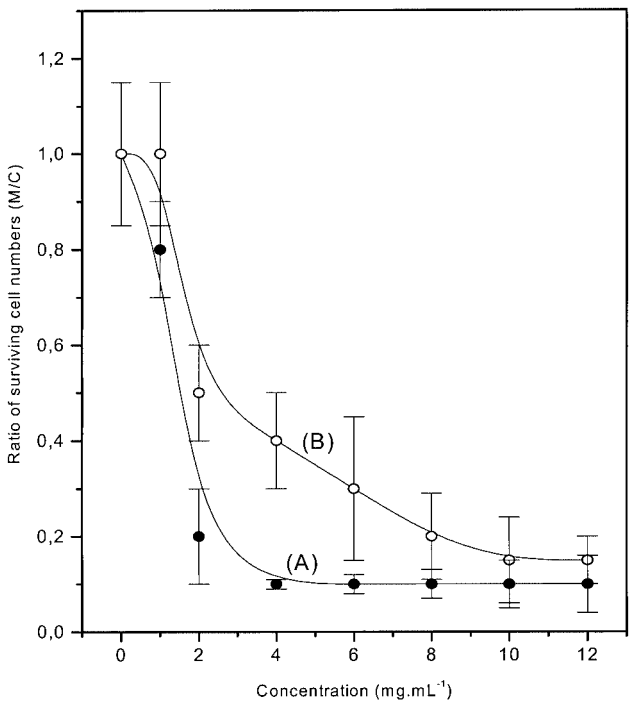


**Figure 7.** Growth inhibition of different concentrations of PCL-I. Inoculation: $6 \times 10^4$ cells · $mL^{-1}$, *E. coli* (A), *S. aureus* (B).

the proximity of the melting temperature of PCL which is approximately 60°C.

The potential cytotoxicity was evaluated quantitatively by the cytotoxicity index $IC_{50}$, the dose that produces 50% of cell survival, from the dose-response curves shown in Figure 6. The cytotoxic character was based on the determination of percent of cell survival per dose of component, and the $IC_{50}$ value was extrapolated from the curves of percent cell survival versus dose. The studies with PCL-I extracts demonstrate that the iodophor was noncytotoxic to the CHO cells.

The antimicrobial capacities of PCL-I against *E. coli* and *S. aureus* were explored by the cut plug method and viable cell counting methods. The capability of the prepared polymer to inhibit the growth of the tested microorganisms on solid media is shown in Figure 7. The growth-inhibiting effect was quantitatively determined by ratio of the surviving cell number (M) in the medium containing the polymer to that without the polymer (C). PCL-I inhibited the growth of the tested bacteria on solid agar medium with increasing concentration of the iodophor. Finally, the results show that the inhibitory effect is more pronounced for *E. coli* and it becomes similar at high concentrations of PCL-I.

## CONCLUSIONS

PCL-I, an essentially macroporous polymeric material exhibiting a rather narrow pore size distribution, was synthesized. The PCL-I macroporous structures revealed no signs of cytotoxicity in the methyl tetrazolium assays, showing a satisfactory biocompatibility. The tested PCL-I samples showed better antimicrobial activity against *E. coli* than against *S. aureus*. Improved antimicrobial PCL-I properties can be explained by a synergistic effect between the polymeric support and the biologically active substance (iodine), linked to the matrix via a charge-transfer complex. However, the exact mode of action of the synthesized PCL-I on the test microorganisms in this study needs more investigation, and such results will be reported in the future.

## References

1. Gristina AG. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 1987;237:1588–1595.
2. Dickinson GM, Bisno AL. Infections with indwelling devices: concepts of pathogenesis infections associated with intravascular devices. Antimicrob Agents Chemother 1989;33:597–601.
3. Leyden JJ, Marples RR, Kligman AM. *Staphylococcus aureus* in the lesions of atopic dermatitis. Br J Dermatol 1974;90:525–530.
4. Williams REA, Gibson AG, Aitchison TC, Lever R, Mackie RM. Assessment of a contact-plane sampling technique and subsequent quantitative bacterial studies in atopic dermatitis. Br J Dermatol 1990;123:493–501.
5. Akiyama H, Ueda M, Toi Y, Kanzaki H, Tada J, Arata J. Comparison of the severity of atopic dermatitis lesions and the density of *Staphylococcus aureus* on the lesions after antistaphylococcal treatment. J Infect Chemother 1996;2:70–74.
6. Shelanski S. PVP-iodine, history, toxicity and therapeutic uses. J Int Coll Surg 1956;25:727–735.
7. Zamora JL. Chemical and microbiologic characteristics and toxicity of povidone-iodine solutions. Am J Surg 1986;151:400–406.
8. Keverline MR, Kowalski RP, Dhaliwal DK. *In vitro* comparison of ciprofloxacin, ofloxacin, and povidone-iodine for surgical prophylaxis. J Cataract Refract Surg 2002;28:915–916.
9. Sinha R, Agarwal RK, Agarwal M. Povidone iodine plus neosporin in superficial burns: a continuing study. Burns 1997; 23(7–8):626–628.
10. Kovacikova L, Kunovsky P, Skrak P, Hraska V, Kostalova L, Tomeckova E. Thyroid hormone metabolism in pediatric cardiac patients treated by continuous povidone-iodine irrigation for deep wound infection. Eur J Cardiothorac Surg 2002;21: 1037–1041.
11. Kubo M, Sakuraba T, Arai Y, Nakazawa M. Dacryocystorhinostomy for dacryocystitis caused by methicillin-resistant *Staphylococcus aureus* report of four cases. Jpn J Ophthalmol 2002;46:177–182.
12. Ibrahim AIA, Rashid M. Comparison of local povidone-iodine antisepsis with parenteral antibacterial prophylaxis for prevention of infective complications of TURP: a prospective randomized controlled study. Eur Urol 2002;41:250–256.
13. Alfonso EC, Flynn HW. Controversies in endophthalmitis prevention: the risk of emerging resistance to vancomycin. Arch Ophthalmol 1995;113:1369–1370.
14. Naor J, Savion N, Blumenthal M, Assia EI. Corneal endothelial cytotoxicity of diluted povidone-iodine. J Cataract Refract Surg 2001;27:941–947.
15. Khera SY, Kostyal DA, Deshmukh N. A comparison of chlorhexidine and povidone-iodine skin preparation for surgical operations. Curr Surg 1999;56(6):341–343.
16. Nahlieli O, Baruchin AM, Levi D, Shapira Y, Yoffe B. Povidone-iodine related burns. Burns 2001;27:185–188.

17. Fabreguette A, Zhi HS, Lasne F, Damour O. Evaluation of the cytotoxicity of antiseptics used in current practice on cultures of fibroblasts and keratinocytes. Pathol Biol 1994;9:888–892.
18. Cooper ML, Laxer JÁ, Hansbrough JF. The cytotoxic effects of commonly used topical antimicrobial agents on human fibroblasts and keratinocytes. J Trauma 1991;31(6):775–884.
19. Smoot EC, Kucan JO, Roth A, Mody N, Debs N. *In vitro* toxicity testing for antibacterials against human keratinocytes. Plast Reconstr Surg 1991;87(5):917–924.
20. Schwope AD, Wise DL, Sell KW, Skornik WA, Dressler DP. Development of a synthetic burn covering. Trans Am Soc Artif Intern Organs 1974;20A:103–106.
21. Schwope AD, Wise DL, Sell KW, Dressler DP, Skornick WA. Evaluation of wound-covering materials. J Biomed Mater Res 1977;11(4):489–502.
22. Khor HL, Ng KW, Schantz JT, Phan TT, Lim TC, Teoh SH, Hutmacher DW. Poly(ϵ-caprolactone) films as a potential substrate for tissue engineering an epidermal equivalent. Mater Sci Eng C 2002;20:71–75.
23. De Queiroz AAA, França EJ, Abraham GA, San Roman J. Ring-opening polymerization of ϵ-caprolactone by iodine charge-transfer complex. J Polym Sci Part B Polym Phys 2002; 40:714–722.
24. Sing KSW. Adsorption methods for the characterization of porous materials. Adv Colloid Interface Sci 1998;76–77:3–11.
25. Remy MJ, Coelho ACV, Poncelet G. Surface area and microporosity of 1.8 nm pillared clays from the nitrogen adsorption isotherm. Microp Mater 1996;7:287–297
26. Dubinin MM. Physical adsorption of gases and vapor in micropores. Prog Surf Membr Sci 1975;9:1–70.
27. Pridham TG, Lindenfelser LA, Shotwell OL, Stodola F, Benedict RG, Foley C, Jacks PW, Zaumeyer WJ, Perston WH, Mitchell JW. Antibiotics against plant disease. I. Laboratory and greenhouse survey. Phytopathology 1956;46:568–575.
28. Kenawy ER, Abdel-Hay FI, El-Shanshoury AERR, El-Newehy MH. Biologically active polymers: synthesis and antimicrobial activity of modified glycidyl methacrylate polymers having a quaternary ammonium and phosphonium groups. J Control Release 1998;50:145–152.
29. Thrupp LD. Susceptibility testing of antibiotics in liquid media. In: Larian V, editor. Antibiotics in laboratory medicine. Baltimore, MD: Williams & Wilkins; 1986. p 57–65.
30. ISO (1992). Biological evaluation of medical devices. Part 5. Tests for cytotoxicity: *in vitro* methods. ISSO document 10993.
31. Rogero SO, Higa OZ, Saiki M, Correa OV, Costa I. Cytotoxicity due to corrosion of ear piercing studs. Toxicol In Vitro 2000; 14:497–504.
32. Abraham GA, Gallardo A, Lozano AE, San Román J. Caprolactone/$ZnCl_2$ complex formation and ring-opening polymerization mechanism. J Polym Sci Part A Polym Chem 2000;38(8): 1355–1365.
33. Sing KSW, Everett DH, Haul RHW, Moscou L, Pierotti RA, Rouquerol J, Siemieniewska T. Reporting physisorption data for gas/solid systems with special reference to the determination of surface area and porosity. Pure Appl Chem 1985;57: 603–619.