Micro-Encapsulation and Antifouling Coatings: Development of Poly(lactic acid) Microspheres Containing Bioactive Molecules

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Summary: Environmental legislation compels marine paints companies to develop non toxic antifouling coatings respecting ecosystems. In this work, biodegradable polymers are used to conceive delivery systems with a lifetime of many months. For this purpose, chlorhexidine was encapsulated in poly(L-lactide) microspheres and incorporated in antifouling formulations. The characterization (encapsulation yield, surface morphology, particle size) and antibacterial activity (bacteriostatic and bactericidal effects) of microspheres were carried out by using scanning electronic (SEM) and confocal laser scanning microscopies (CLSM). The results indicate a good ability of loaded microspheres to be formulated even though an excellent activity against selected marine bacteria is conserved. This is a promising approach to develop biodegradable antifouling paints based on non toxic molecules and bioactive surfaces.

Keywords: coatings; controlled release; microencapsulation; microscopy; poly(lactic acid)

Introduction

Marine biological fouling, usually termed marine biofouling, can be defined as the undesirable accumulation of microorganims, plants and animals on immersed surfaces. Biofouling can increase dramatically both economic and environmental costs (high fuel consumption, emissions of harmful compounds, deterioration of coating such as corrosion, introduction of invasive species...). Since the early 1970s, several preventing systems have been developed in order to prevent the development of biofouling.[1,2] At the moment, antifouling paint is realized by the blending of poly(methylmethacrylate-co-butylmethacrylate) resins (PMMA-PBMA) with two types of biocides: organic biocides (Zinc pyrithione, tolylfluanid, diuron) and mineral biocides such as cuprous oxide.[1] However, these molecules are toxic to other non-target organisms and their accumulation in the aquatic environment has recently been a topic of increasing concern.[3] These findings have created a considerable interest to produce a new generation of protective systems based on non toxics molecules.[4] Preliminary to this work, it is needed to better understand the mechanisms leading to antifouling activity. It has been proven that two main properties are involved: erosion and biocide availability at film surface.[5] The erosion can be controlled by modelling biodegradable polymers with specific chemical functions such as polyester or poly(ester-anhydride). The presence of biocide in the paint film depends on the distribution of the particles in the film i.e. on the formulation.

In this study we have tested an antibacterial molecule, called chlorhexidine. It is a bisdiguanide antiseptic widely used in dentistry as an anti- dental plaque agent and has demonstrated good antibacterial activity against a wide range of bacteria.[6]

However, previous works have shown that chlorhexidine as many antifouling molecules (particularly natural ones) was too rapidly released.[1,4,6,7] These findings

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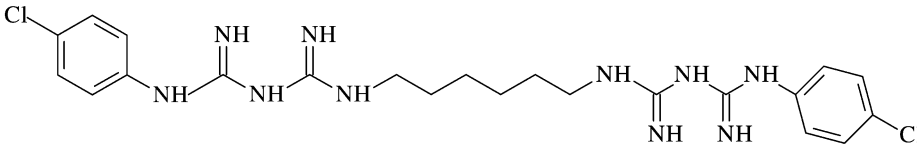

Chlorhexidine

implicate to develop its microencapsulation in order to control the leaching.[8,9] Consequently, the aim of the present study was to investigate the potential of active molecules entrapped into poly(L-lactide) (PLA) microparticles to provide sustained bacterial activity against marine bacteria.

This study presents i) the encapsulation of chlorhexidine with a biodegradable polymer (PLA), prepared by the solvent evaporation method ii) the characterization of the obtained microparticles: size, morphology and encapsulation efficiency were studied iii) the influence of encapsulation on the activity of chlorhexidine (growth and viability of marine bacteria), iiii) the incorporation of microparticles in antifouling coatings.

Materials and Methods

Materials

Chlorhexidine, polyvinylalcohol (PVA) (MW 30 000, 88% hydrolyzed) and poly-(lactic acid) (PLA) (MW 100 000) provided by Sigma-Aldrich. Sytox Green (S7020) and Syto Red 61 (S11343) were purchased from Molecular Probes. All other chemical reagents were obtained from Fisher Bioblock.

Microparticles Preparation

The microencapsulation of chlorhexidine which is a hydrophobic compound has been realized by oil/water emulsion-solvent evaporation method.[10] Briefly, 200 mg PLA (100 000 g/mol) and 40 mg chlorhexidine were dissolved in 10 mL dichloromethane. The resultant organic solution was added to 100 mL PVA used as surfactant (1%w/v) and emulsified by using a homogenizer (Polytron PT-MR 2100, Fisher Bioblock) at 13,000 rpm for 1 min. The system was stirred continuously for 3 h at room temperature and atmospheric pressure to evaporate the dichloromethane completely. The particles were centrifuged, washed three times with distilled water and freeze-dried.

Characterization of the Particles

The external morphology of microparticles was analysed by a JEOL 6460 LV scanning electron microscopy (SEM). Samples were mounted on metal stubs and coated with gold.

Analysis of the chlorhexidine was carried out by EDX analysis using an OXFORD INCA 300 system. Chlorhexidine was observed from chlorine.

Determination of Loading, Encapsulation Efficiency and Chlorhexidine Release

The amount of encapsulated load in microparticles was determined by dissolving 3 mg of microparticles in 1 mL dichloromethane. Then the solvent was allowed to evaporate. The residue was reconstituted in 5 mL of the mixture of acetonitrile/water (1:1 v:v) for UV- analysis (spectrophotometer Perkin Elmer Lambda 25). The detection was carried out at 260 nm.

The amount of chlorhexidine release in surrounding medium was determined by suspending 3.5 mg of microspheres in 5 mL of distilled water at 20 °C with shaking. After 24 hours, the degradation medium was centrifuged and surrounding water was analysed by UV spectrophotometer.

Antibacterial Activity Study

The marine bacterium, a *Pseudoalteromonas* of our collection, was grown on a rich medium, Marine Broth (Bacto Marine

Broth 2216, Difco) at 20 °C with shaking. The bacterial growth inhibition studies were carried out in bacterial solution by incorporating active molecules at an optical density of 0.1 (at $\lambda = 600$ nm). To promote the contact molecules-bacteria, a gentle agitation was achieved.

A TCS SP2 Laser Scanning Confocal microscope (Leica Microsystems) was used to investigate antibacterial activity. The bacterial growth was measured by CLSM after coloration by two fluorescent Nucleic acid stains: syto 61 (20 μM) and sytox green (0,25 μM) after 3, 8 and 24 h. The fluorescence excitation/emission wavelengths were 633/640–800 and 488/500–550 respectively. The viability studies were realized by incorporating the active molecules after 7 hours of culture (beginning of stationary phase, $OD_{600\ nm} = 1.4$). After 15 hours of incubation at 20°C, live and dead bacteria were counted by the ''Image tools'' software.

Results and Discussion

Characterization of PLA Microparticles

Bioactive microspheres prepared by using the emulsion-solvent evaporation method were characterised. The efficiency of the encapsulation process was tested for a nominal loading of 20% of polymer (w/w). Loading and yield were defined as following:

- loading = mass of chlorhexidine in microspheres/mass of microspheres;
- yield = mass of microspheres/(mass of chlorhexidine + mass of polymer)$_{initial}$.

The loading efficiency was expressed as the amount of the chlorhexidine in microspheres divided by the weight of chlorhexidine used for formulation.

Results show that encapsulation efficiency of chlorhexidine in PLA microspheres was 85% versus the initial chlorhexidine amount dissolved in the organic mixture. The chlorhexidine loading in PLA microspheres was 17% (w/w). Moreover, the yield of microspheres was high at 80%.

Figure 1 shows the surface structure of the prepared microspheres. It can be seen that the majority of the particles were spherical with smooth surfaces and without visible pores. The average diameter of the microspheres was 1 μm. However, an heterogeneity of microparticles (nano and microparticles) was observed. The chlorhexidine entrapment efficiency in PLA microparticles was confirmed by X-ray microanalysis which is based on chlorine detection.

Biological Activity

The antibacterial activities of chlorhexidine and microspheres against *Pseudoalteromonas,* a Gram-negative bacterium, was then evaluated in suspension. SYTO 61 has been used with SYTOX Green to image live and dead bacteria by CLSM. SYTO 61 is cell-permanent nucleic acid stains, whereas SYTOX green is a high-affinity nucleic acid stain that

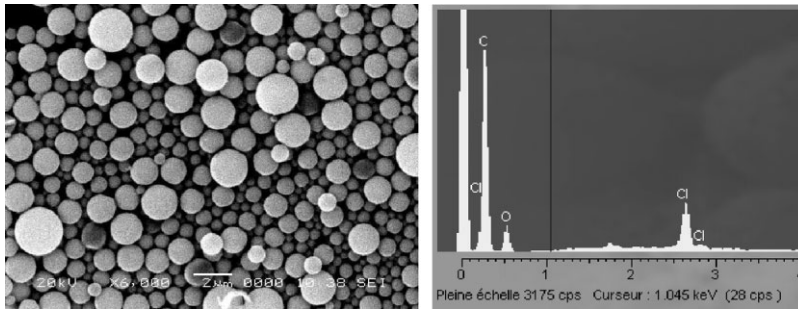

Figure 1.
SEM photograph of microspheres loaded by chlorhexidine and corresponding X-ray spectrum.

easily penetrates cells with compromised plasma membranes and yet will not cross the membranes of live cells. After incubation with SYTO/SYTOX, live and dead bacteria are easily distinguished by CLSM: the nucleic acids of live and dead cells are stained in red and green respectively.

The influence of chlorhexidine (without prior encapsulation) on the growth was studied at several concentrations (1 μg/L, 1 mg/L and 100 mg/L). The Figure 2 illustrates the ability of bacteria to adapt to the changing environment for their survival. In unfavourable conditions, micro-colonies were observed (Figure 2 B and C). Nevertheless, the minimal bactericidal concentration of chlorhexidine obtained in solution was relatively high 100 mg/L for *Pseudoalteromonas* (Figure 2 D). For this concentration, the few bacteria that are observed are dead.

Microspheres were realised from a chlorhexidine solution at a concentration of 700 mg/L in order to take into account the loading efficiency. In these conditions, the amount of active molecules present in the particles is around 100 mg/L. The results presented in Figure 3 and 4 illustrate the effect of encapsulation on the activity of chlorhexidine.

The control microspheres without chlorhexidine was slightly active towards the bacterial strain: a growth decrease seems to be observed (Figure 4(B)). However, no effect on the viability was distinguished (Figure 3(B)). Both chlorhexidine and encapsulated chlorhexidine kill bacteria through cell membrane damages: the cell mortality was 80 and 85% after 15 hours of contact respectively (Figure 3(C,D)). Moreover, according to antibacterial activity study results presented in Figure 4, these microspheres inhibited the *Pseudoalteromonas* growth (Figure 4(C,D)). This activity should be explained by the release profile of

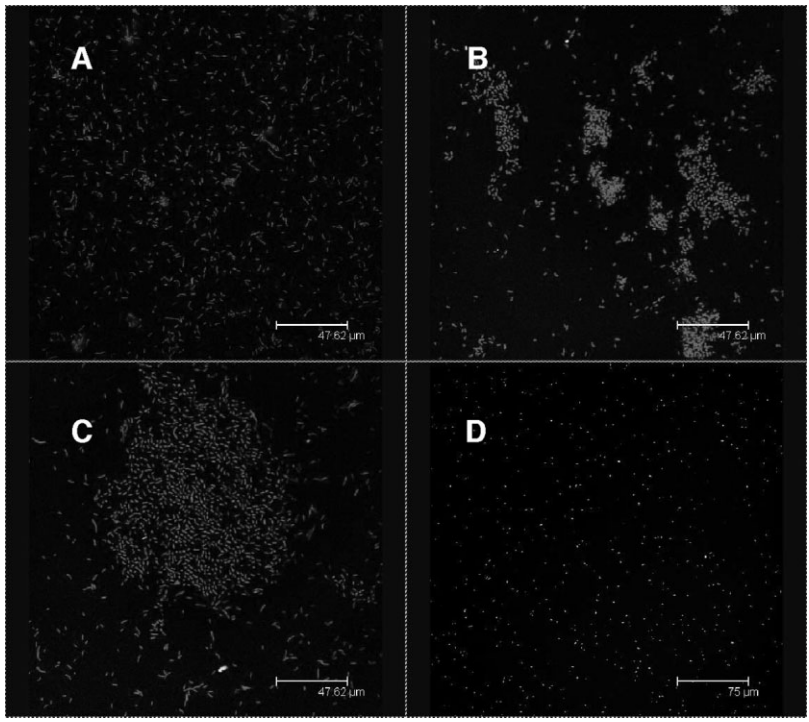

Figure 2.
Antibacterial activity of chlorhexidine against *Pseudoalteromonas*. A. without chlorhexidine, and in presence of chlorhexidine B. 1 μg/L, C. 1 mg/L, D. 100 mg/L.

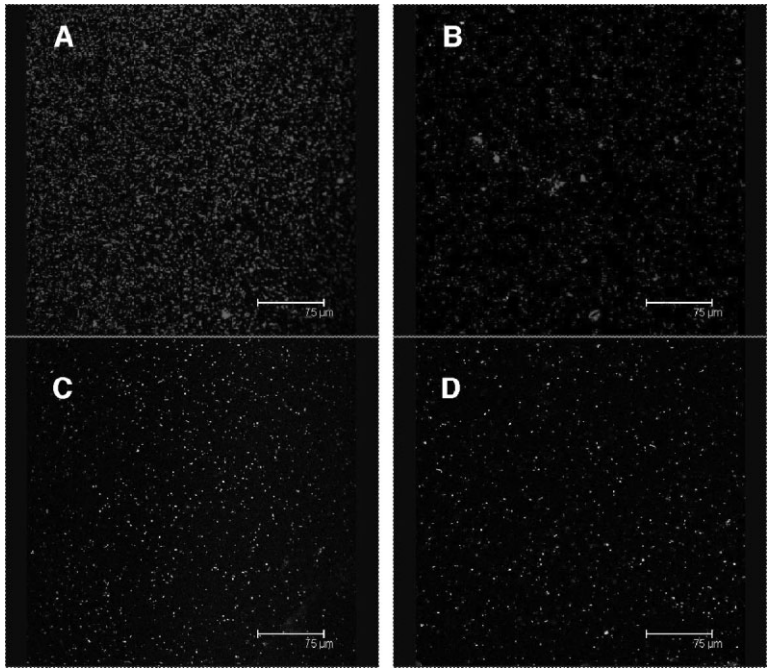

Figure 3.
CLSM of *Pseudoalteromonas* after 15 h of contact with encapsulated substances. A. Without molecule, B. Microspheres without bioactive molecules (700 mg/L), C. Chlorhexidine (100 mg/L), D. Microspheres loaded with chlorhexidine (700 mg/L).

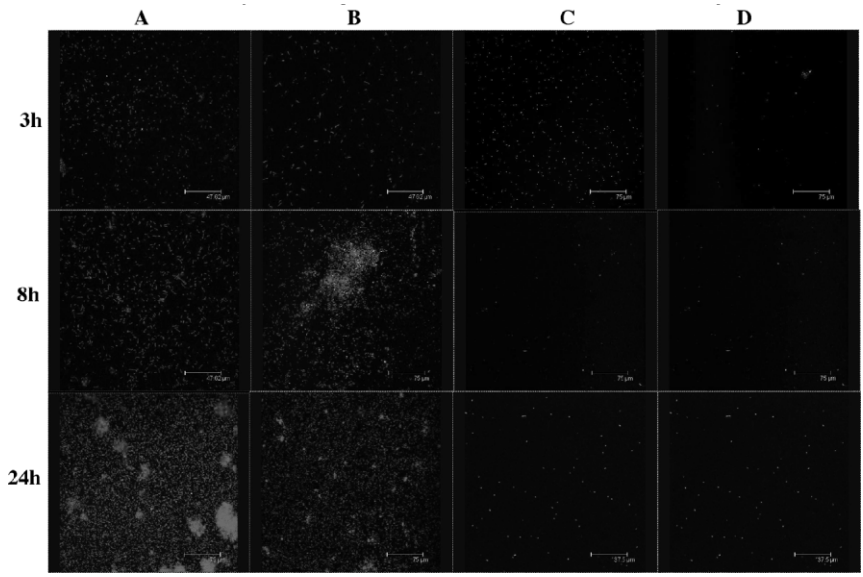

Figure 4.
CLSM of Pseudoalteromonas after 3, 8 and 24 h of culture with bioactive molecules. A. Without molecule, B. Microparticles without bioactive molecules (700 mg/L), C. Chlorhexidine (100 mg/L), Microparticles loaded with chlorhexidine (700 mg/L).

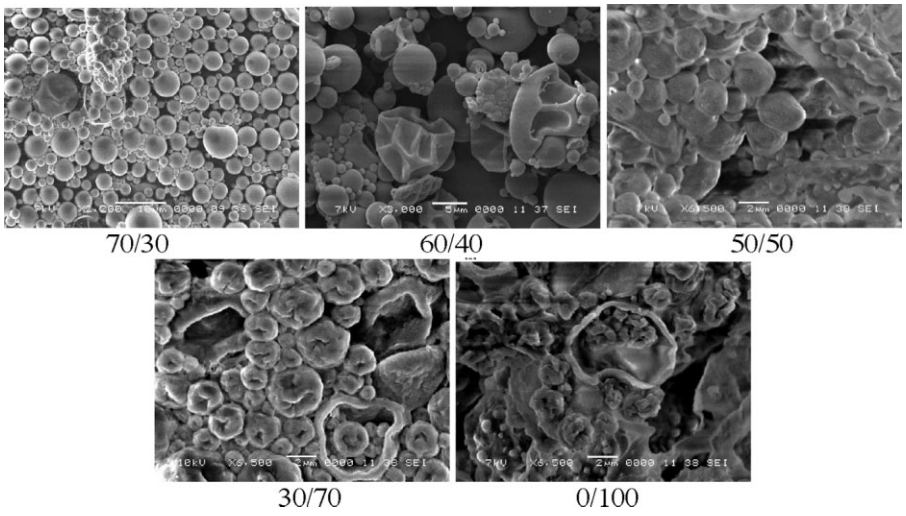

Figure 5.
SEM photographs of microspheres in isopropylic alcohol/xylene blend.

microspheres. For this purpose, microparticles were placed in deionised water under agitation. The quantification of released molecules was realised by UV analysis of the surrounding water. The leaching is 39% after 24 hours. The important release may be explained by the burst effect which is generally observed for microspheres. Further quantification will be carried out to confirm that the leaching will reach a steady state after many days of immersion. Nevertheless, all these experiments demonstrate that the encapsulation method used enables to keep the biological activity of the chlorhexidine molecules.

Bioedegradable Coating Based on Microspheres Development

In a previous study, we have developed biodegradable coatings based on poly(ε-caprolactone-co-δ-valerolactone) (P(CL-VL)).[11] Antifouling coating was based on equal weights of P(CL-VL) and diluting agent such as xylene and on the incorporation of active molecules.

The presence of diluting agent must not modify the morphology of microspheres by degradation or diffusion process. However, as illustrated in Figure 5(E), xylene dramatically damages PLA microspheres. The particles were deformed and their surfaces showed cracks.

In order to determine the suitable diluting agent, i.e. a P(CL-VL) solvent and a PLA microsphere non-solvent, microspheres were placed in a mixture of isopropylic alcohol and xylene (Figure 5). The increase of isopropylic alcohol percentage in the melt limits the degradation of microspheres. The best mixture contains 70% of isopropylic alcohol and 30% of xylene (Figure 5(A)). Coatings were formulated by dispersion of P(CL-VL) polymer and encapsulated chlorhexidine in PLA in this binary solvent.

Conclusion

Chlorhexidine loaded PLA microspheres were successfully prepared by emulsion-evaporation process. Through a number of tests, it was proven that chlorhexidine loaded PLA microspheres showed antibacterial activity against *Pseudoaltermonas*. These biodegradable coating based on PLA microspheres are a promising novel antibacterial material. The results enable to enlarge the use of PLA and chlorhexidine from the medical field to environmental applications. Future works include the

profound investigation of these biodegradable coatings through different studies such as physico-chemical characterization (hydration, degradation, erosion and chlorhexidine release), in vitro (influence on the growth, viability and adhesion) and in situ (tests on a natural marine site) microbiological evaluations.

[1] D. M. Yebra, S. Kiil, K. Dam-Johansen, *Prog Org Coat* **2004**, *50*, 75.
[2] E. Almeida, T. C. Diamantino, O. de Sousa, *Prog Org Coat* **2007**, *59*, 2.
[3] I. K. Konstantinou, T. A. Albanis, *Environ Int* **2004**, *30*, 234.
[4] L. D. Chambers, K. R. Stokes, F. C. Walsh, R. J. K. Wood, *Surface & Coatings Technology* **2006**, *201*, 3642.
[5] F. Faÿ, I. Linossier, J. J. Peron, V. Langlois, K. Vallée-Réhel, *Prog Org Coat* **2007**, *60*, 194.
[6] N. J. Medlicott, I. G. Tucker, M. J. Rathborne, D. W. Holborow, D. S. Jones, *Int. J. Pharmaceutics* **1996**, *143*, 25.
[7] R. de Nys, P. D. Steinberg, *Current Opinion in biotechnology* **2002**, *13*, 244.
[8] R. P. Price, M. Patchan, A. Clare, D. Rittschof, J. Bonaventura, *Biofouling* **1992**, *6*, 207.
[9] M. Edge, N. S. Allen, D. Turner, J. Robinson, K. Seal, *Prog Org Coat* **2001**, *43*, 10.
[10] P. B. O'Donnell, J. W. McGinity, *Adv Drug Delivery Reviews* **1997**, *28*, 25.
[11] F. Faÿ, I. Linossier, V. Langlois, E. Renard, Vallée.-Réhel. Karine, *Biomacromolecules* **2006**, *7*, 851.

