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# Controlled Release Carriers of Growth Factors FGF-2 and TGF $\beta$ 1: Synthesis, Characterization and Kinetic Modelling

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The purpose of this work is to produce microspheres loaded with transforming growth factor  $\beta$ 1 TGF $\beta$ 1 and basic fibroblast growth factor FGF-2; to ensure the protein protection from degradation during the encapsulation and storage steps, to evaluate the release rate and the microspheres toxicity. The water in oil in water double emulsion technique was adapted to avoid the protein degradation during the encapsulation. The obtained microspheres were deeply characterized to evaluate their size, morphology, toxicity, the way of degradation, the protein stability and release rate. The microspheres were found to be biocompatible and the encapsulation efficiency was about 35%. It was observed that the obtained microspheres increase the shelf life of the growth factors. The diffusion coefficient was quantified using Fick's law of diffusion that was combined to an empirical equation representing the decrease in the protein stability. Such modelling helped to give indirect information about the microspheres morphology and drug distribution within the microspheres. The main conclusion consists of the formation of a higher compact polymer matrix when smaller particles are produced, which has different distinct effects: the encapsulation efficiency and the stability of the encapsulated growth factor are enhanced while both the growth factor diffusion and the polymer degradation rates decrease.

**Keywords:** Polymers of Lactide and Glycolide, Growth Factors, Microencapsulation, Controlled Drug Release.

## 1. INTRODUCTION

Growth factors have a central role regulating a variety of cellular processes (proliferation, migration, differentiation...). Due to their short half life in solution, notable efforts were made to provide longer-term release of growth factors in tissue engineering and to ensure appropriate therapeutic concentrations. In a first approach, scaffolds,<sup>1</sup> collagen sponge or agarose beads<sup>2</sup> were directly impregnated with growth factors and applied for tissue regeneration. In a second approach, the growth factors were encapsulated in colloidal microspheres of micron size.<sup>3</sup> Studies also reported microspheres embedded in a gel and used in a

scaffolds system.<sup>1,4</sup> In some cases, the microspheres were produced, sterilized then loaded with the growth factor and lyophilized.<sup>5</sup> In this case, the growth factor is adsorbed on the polymer matrix. Encapsulation of growth factors ensures a better protection and controlled release than posterior incorporation to preformed spheres which provides a sustained effect. Advantages of using encapsulation methods are also related to the possibility of characterizing their physicochemical properties and relating them to the release rate.

A number of microencapsulation techniques have been developed and reported to date such as stimuli-responsive microgel containing protein,<sup>6</sup> single emulsion process,<sup>7,8</sup> double (multiple) emulsion process, phase separation (coacervation), spray drying<sup>9</sup> and nanoprecipitation.<sup>10,11</sup>

The choice of the technique depends on the nature of the polymer, the drug, the intended use, and the duration of the therapy. Due to their hydrophilic nature, growth factors (also some proteins and peptides) are usually encapsulated by the water-in-oil-in-water (w/o/w) method followed by solvent extraction/evaporation.<sup>12–15</sup> This microspheres fabrication procedure allows a better protection of the growth factor against degradation since it reduces the contact between the encapsulated agent and the organic solvent.

Protective biomaterials used for encapsulation of such drugs are mainly biodegradable natural polymers as dextrans,<sup>16</sup> chitosan<sup>17</sup> hyaluronic acid, and biodegradable synthetic polymers as polycaprolactone (PCL)<sup>18</sup> and polymers of lactide and glycolide (PLGA).<sup>19,20</sup> PLA, PGA and their copolymers have been used to form both scaffolds and microspheres. The use of PLGA is approved by the U.S FDA.<sup>19</sup> These copolymers have been used to prepare various drug loaded devices (vaccines, peptides, proteins and micromolecules) due to their excellent biocompatibility and biodegradability.

In this work, PLGA is used to encapsulate FGF-2 or TGF $\beta$ 1 using the w/o/w method. The produced microspheres were deeply characterized to investigate their toxicity, degradation rate of polymer, microspheres morphology, stability of the encapsulated drug, drug distribution, encapsulation efficiency, and the drug release rate. A mathematical law is used to describe the release rate. The combination of physical measurements and modelling estimations was found to be beneficial to investigate this system and interpret some observations.

## 2. MATERIALS AND METHODS

### 2.1. Materials

Poly (D,L lactic-co-glycolic acid) (PLGA) is RESOMER<sup>®</sup> RG 502H with a copolymer lactide-glycolide ratio of 48:52 to 52:48 was purchased from Boehringer Ingelheim. Recombinant Human Transforming Growth Factor-beta 1 (TGF $\beta$ 1) (25 KDa) was purchased from AbCys Company/France. Recombinant Human Fibroblast Growth factor FGF-2 (17 KDa) was kindly provided by Whenzhou Medical College/China. Albumin–fluorescein isothiocyanate conjugate bovine (FITC–BSA) (60 kDa) was purchased from Sigma Chemical. Poly(vinyl alcohol) (PVA) was obtained from Fluka and Methylene chloride (DCM) from Carlo Erba Reagents. RayBio<sup>®</sup> Human

TGF $\beta$ 1 and FGF-2 enzyme-linked immunosorbent assay (ELISA) Kit was purchased from BioCat/Germany. Cell culture medium used for stability and release studies was a serum-free DMEM (Gibco/France) supplemented with micokill and ciprofloxacin (Bayer/France).

### 2.2. Preparation of Microspheres

Microspheres of PLGA were prepared by the w/o/w double-emulsion and solvent extraction/evaporation method as previously described<sup>14,15</sup> (Table I). PLGA was dissolved in DCM. This oil phase was then emulsified using a high speed mixing apparatus (Ultrathurrax<sup>®</sup>, T25 basic, IKA<sup>®</sup> Werke/Germany) with an internal aqueous phase phosphate saline buffer (PBS) solution containing TGF $\beta$ 1 or FGF-2 always with FITC-BSA to form a w/o emulsion. All preparations were performed at ambient temperature. The resulting emulsion was added to 50 mL of external aqueous solution containing 0.1% (w/v) PVA and emulsified with Ultrathurrax<sup>®</sup> in order to produce the double w/o/w emulsion. The double emulsion was then poured into a large volume of water (100 mL) under magnetic stirring for 2.5 hours to allow removal of the organic solvent. Finally, the resulting microspheres were collected on a filter, washed twice with 50 mL of deionised water, dried and stored at  $-20^{\circ}\text{C}$ . Table II shows the different operating conditions considered to investigate the microspheres morphology.

The main precautions included in the previously developed w/o/w encapsulation method are:

—Double sterilized water was used in all solutions; beakers and glasses were sterilized; preparations take place under fume hood.

—Growth factors were stored at  $-20^{\circ}\text{C}$ ; Growth factor solutions were prepared 10 min before the emulsion preparation.

—Stirring time of the first w/o emulsion was limited to 30 s to avoid heating.

—For solvent extraction, the final solution was poured into a large quantity of water under magnetic stirring without vacuum evaporation to avoid pressure aggression and temperature rising. Also, addition of isopropanol alcohol to accelerate solvent extraction was avoided.

—For protein extraction, Dimethyl sulfoxide (DMSO) was used to dissolve the microspheres, which is less aggressive than DCM used previously for PLGA dissolution.<sup>21,22</sup>

**Table I.** Conditions of the double emulsion method.

Growth factor	Internal aqueous phase	Oil phase	First emulsion stirring time	First emulsion stirring speed	External aqueous phase	Second emulsion stirring time	Second emulsion stirring speed
FGF-2	PBS solution containing 1 mg FITC-BSA and 5 $\mu\text{g}$ FGF-2	2 mL methylene chloride containing 500 mg PLGA	30 s	13000 rpm	0.1% (w/v) PVA	30 s	6500 rpm
TGF $\beta$ 1	PBS solution containing 500 $\mu\text{g}$ FITC-BSA and 1.5 $\mu\text{g}$ TGF $\beta$ 1						

**Table II.** Conditions of the different experiments with the resulting microspheres size and encapsulation efficiency.

Experiment	Active agent	Internal aqueous phase volume ( $\mu\text{L}$ )	Water to oil volume ratios	Second emulsion stirring speed	Stirring apparatus	Size ( $\mu\text{m}$ )	Encapsulation efficiency Method 1	Encapsulation efficiency Method 2	Loading ng/mg (Method 1)
1a	FGF-2	250	1/2	6500	ULTRA-TURRAX <sup>®</sup>	8 $\pm$ 5	32	35.3	2.9
1b	TGF $\beta$ 1					11 $\pm$ 5	33	36.3	1
2a	FGF-2	100	1/5			8 $\pm$ 5.5	32.4	35.3	2.9
2b	TGF $\beta$ 1					11 $\pm$ 5	30.1	34.4	0.9
3a	FGF-2	25	1/10			8 $\pm$ 6.2	31.3	35.3	2.8
3b	TGF $\beta$ 1					11 $\pm$ 5	31.7	38.9	0.95
4a	FGF-2	100	1/5	2000	Ika <sup>®</sup> Mechanical Overhead Stirrers	93 $\pm$ 25	24.3	28.	2.2
4b	TGF $\beta$ 1					137 $\pm$ 40	23.5	28.1	0.71

### 3. MICROSPHERES CHARACTERIZATION

#### 3.1. Particle Size and Morphology

Scanning electron microscopy (SEM) was performed using a FEG Hitachi<sup>®</sup> S 800 microscope. Microspheres were mounted onto metal stubs with a double sided adhesive tape, vacuum-dried, contacted with silver paint, sputter-coated with a thin layer of gold (10–150Å) and imaged with the SEM at 15 kV or 10 kV. The size distribution was determined with a laser diffraction technique using a Coulter<sup>®</sup> counter multisizer (Beckman Coulter LS 230) after dispersion of the microspheres in deionised water.

#### 3.2. Enzyme-Linked Immuno Sorbent Assay

The titration of growth factors was performed using a human ELISA kit specific for each growth factor. 100  $\mu\text{L}$  of each sample solution were added into appropriate wells of the kit microtiter plate. After 2.5 hours of incubation at room temperature, the plate was rinsed several times with the kit buffer solution and a biotinylated antibody solution was added into each well and incubated for 1 h. After washing away the unbound biotinylated antibody, a Streptavidin solution was added to the plates and incubated for 45 min. The plates were finally washed five times and introduced into a luminescent plate reader (LabSystem). The light emission was recorded after the injection in each well of 150  $\mu\text{L}$  buffer containing 200  $\mu\text{M}$  of luminol, 500  $\mu\text{M}$  of hydrogen peroxide and 200  $\mu\text{M}$  of p-iodophenol. The calibration curves were generated for each growth factor with the appropriate kit standard solutions ranging from 0 to 100 ng/mL.

#### 3.3. Encapsulation Efficiency and Drug Loading

Two methods were used to measure the encapsulation efficiency. Method (1) consists of measuring the amount of growth factor entrapped in the microspheres after extraction (extraction protocol). The extraction protocol consists of dissolving about 20 mg of microspheres in 1 mL of DMSO then adding 9 mL of cultural medium and analysing by ELISA. In Method (2), the encapsulation efficiency was calculated by deducting the lost quantity of

growth factors in the aqueous supernatant at the end of the microspheres preparation (after solvent evaporation, before rinsing) from the initial used quantity.

#### 3.4. Protein Distribution into the Microspheres

Protein distribution into the microspheres could be analyzed thanks to the presence of FITC–BSA in the microspheres, by confocal laser scanning microscopy (Leica Microsystems TCS SP2/Germany). The microspheres were suspended into water and spread on a cover slip. The fluorescein was excited by a 488 nm argon laser. Different sections of the microspheres were scanned. The images presented in this work were taken in a central section of the microspheres.

#### 3.5. Residual Solvent

Gas chromatography was used to analyze the residual amount of DCM (Boiling point (BP) = 40 °C). An Agilent Model 4890 gas chromatograph was used with the program Star Chromatography Workstation and a BONDED FSOT Capillary column 30 m  $\times$  0.53 mm (i.d.). The analytical conditions were: injector temperature 250 °C; detector temperature, 280 °C; initial oven temperature 70 °C that increased at 10 °C/min to 220 °C with a final stabilization at this temperature for 2 min and flow rate of the carrier gas (nitrogen) was 13 mL/min. The calibration curve was based on different DCM concentrations in DMSO (500–31 ppm) with toluene as internal standard at a constant concentration of 100 ppm then using the ratio of DCM to toluene areas under the peak. For the measurement of DCM residual amount in the microspheres, 50 mg of microspheres were dissolved in 2 mL of DMSO (BP = 189 °C), an appropriate amount of toluene (BP = 111 °C) was added as an internal standard.

#### 3.6. PVA Content

The residual amount of PVA in the microspheres was determined using an iodine–borate colorimetric method as proposed in Ref. [23] including some modifications proposed.<sup>7</sup> The method requires the extraction of

poly(vinyl alcohol) from the polymer matrix into the aqueous phase, followed by the formation of a PVA–iodine–borate complex that can be detected by visible spectroscopy.

### 3.7. Stability of Growth Factors in Aqueous Solution

In order to study the growth factor stability in aqueous PBS solution (pH = 7.4) or in the culture medium (usually used for *in vitro* test on cultured cells), the same amount of growth factor (50 ng) was dissolved in flasks containing 1 mL solution. These flasks were put either at ambient temperature or in the refrigerator and the growth factor content was analyzed using ELISA.

### 3.8. Microspheres Shelf Life

The microcapsules shelf life, or the stability of encapsulated growth factors in the PLGA microsphere matrices, was studied as follows: Flasks of 20 mg of microspheres were put at room temperature or at 5 °C. The growth factor content was analyzed at specific intervals using ELISA by applying the extraction protocol explained above.

### 3.9. Size Exclusion Chromatography (SEC)

PLGA Degradation during the release was studied by monitoring the polymer molecular weight using Size Exclusion Chromatography (SEC). Waters SEC system was used. This system was equipped with an isocratic pump (Waters 515) operating at a flow-rate of tetrahydrofuran (THF) of 1 mL/min, a refractive-index detector Model (Waters 410) with integrated temperature controller to maintain temperature at 35 °C, a guard column (PLgel 5  $\mu$ m) and three Polymer Laboratories columns (2  $\times$  PLgel 5  $\mu$ m Mixed C (300  $\times$  7.5 mm) and 1 PLgel 5  $\mu$ m 500 A (300  $\times$  7.5 mm)), all columns working in-line and the software Empower pro. The calibration was carried out using narrow distributed polystyrene standards. After specific time intervals of suspension in PBS, the microspheres were collected and vacuum dried for 24 h to determine the PLGA molecular weight. Samples of microspheres were dissolved in THF and put in an ultrasonic bath to obtain a homogenous solution. Chromatography was carried out after sample filtration using a 0.45  $\mu$ m filter.

### 3.10. Microspheres Morphology Alteration

The morphology of the microspheres was assessed by SEM and the evolution in the surface properties of the microspheres was analysed by atomic force microscopy (AFM) after specific intervals of suspension in water. For AFM analysis, after suspension in distilled water, the microspheres were taken and deposited on freshly cleaved muscovite mica. The still wet sample was observed at room temperature on a multimode-Veeco AFM in tapping mode.

### 3.11. *In Vitro* Release Study

Known quantities of microspheres were dispersed in test tubes containing 1 mL of culture medium. The suspension was gently stirred at room temperature. At specific intervals, the tubes were centrifuged at a rotating rate of 14000 rpm for 10 min and analyzed by ELISA.

## 4. RESULTS

### 4.1. Microspheres Size and Morphology

As usually observed in the double emulsion method,<sup>14, 15, 21, 22</sup> a broad size distribution was obtained for formulations containing TGF $\beta$ 1 or FGF-2 (Table II). Figure 1 shows that the obtained microspheres are quite spherical and have a smooth and regular surface as observed by SEM and confirms the polydispersity of the microspheres.

The water-to-oil volume ratio in the internal emulsion had no effect on the particle size as previously shown.<sup>15, 21</sup> The stirring speed, on the contrary, had a significant effect on the microspheres size that varies from about 90 to 8  $\mu$ m with stirring speeds of 2000 and 6500 rpm.

### 4.2. Encapsulation Efficiency and Drug Loading

Using the method referred as the extraction protocol, the encapsulation efficiency was 32% for small microspheres and 24% for big ones for both growth factors (Table II). The second method consisting of dosing the growth factor lost in supernatant after collecting the microspheres estimates the encapsulation efficiency between

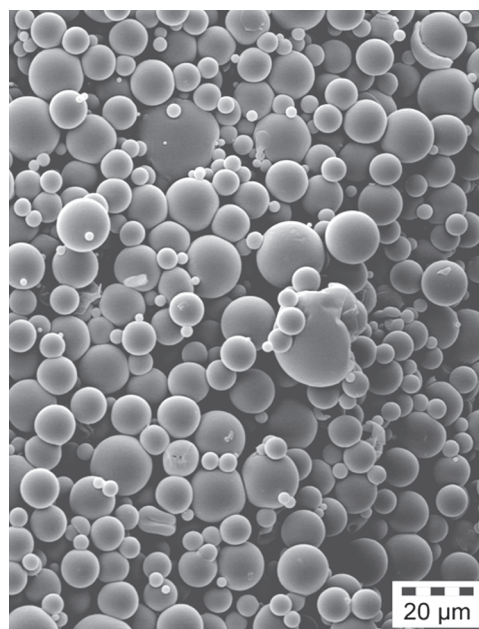


Fig. 1. SEM pictures of PLGA microspheres (experiment 2a).

35% and 39% for small microspheres and approximately 28% for big ones. The difference between both methods can be explained by the reduction of the growth factor activity due to contact with solvent or water. On one hand, during the extraction protocol, the contact with DMSO might reduce the growth factor stability which misestimates the real loading of microspheres. On the other hand, the activity of growth factor present in the aqueous supernatant might decrease and hence the calculation leads to overestimating the real growth factor loading.

From these data, growth factors loading into the microspheres could be calculated and was found to be equal to 2.9 ng/mg for FGF-2 and approximately 1 ng/mg for TGF $\beta$ 1.

#### 4.3. Protein Distribution within the Microspheres

FITC-BSA was incorporated into the microspheres together with the growth factors. The fluorescence of the albumin allows the detection of FITC-BSA in the microspheres using fluorescence confocal microscopy. The distribution of FITC-BSA in the microspheres should be comparable to that of the growth factors, but not necessarily the encapsulation efficiencies. Confocal microscopic images show a homogeneous distribution of the FITC-BSA in the microspheres at 30 min with a slightly higher density near to the surface and in the central part (Fig. 2(a)). The centre contains no FITC-BSA after 1 day (Fig. 2(b)) and after 7 days, FITC-BSA is mainly present in the periphery (Fig. 2(c)). This suggests a gradual diffusion of FITC-BSA through the microsphere with time.

#### 4.4. Residual Solvent

Residual DCM level in the microspheres as determined by GC was always about 3 ppm of DCM per mg of microspheres (about 150 ppm of DCM in 50 mg of microspheres).

#### 4.5. PVA Content

Blank samples (not containing protein or growth factors) were previously analyzed<sup>14</sup> for the PVA content before and after double rinsing and it was found that rinsing allows eliminating most of the PVA. In this work, after rinsing, 0.5% by weight of PVA was found in the microspheres. Similar results were reported in the literature.<sup>9,23</sup>

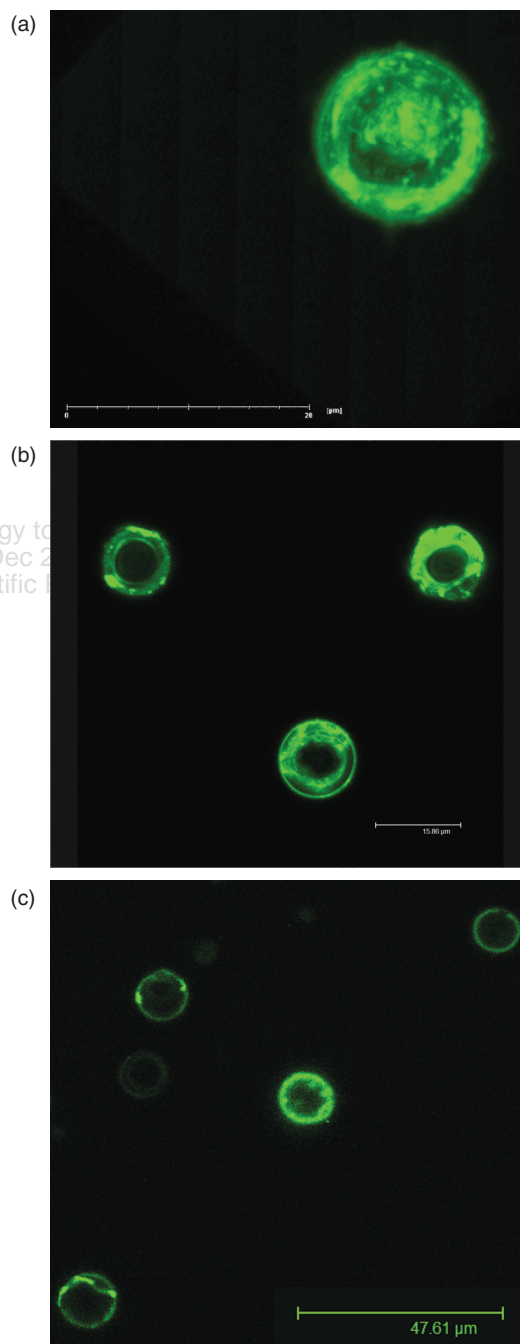
#### 4.6. Growth Factors Stability in Aqueous Solution

The stability of FGF-2 and TGF $\beta$ 1 in PBS buffer (pH 7.4) or in the culture medium was measured by ELISA. The decrease of FGF-2 and TGF $\beta$ 1 concentration with time in the PBS buffer was very fast (Fig. 3). On the other side,

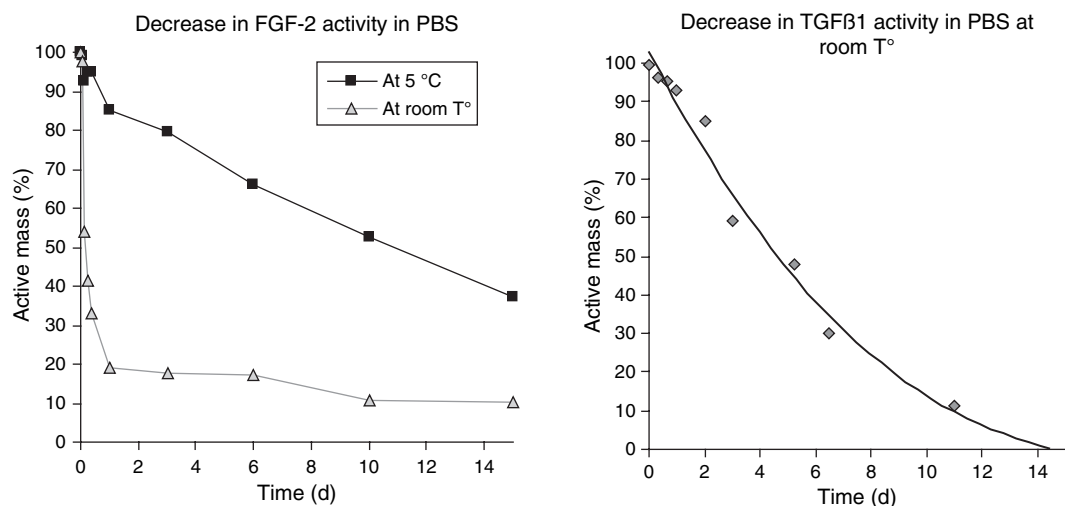
growth factors stability in culture medium was very good. Figure 4 shows only FGF-2 stability. Similar results were found for TGF $\beta$ 1 (data not shown).

#### 4.7. Microspheres Shelf Life

Figures 5 and 6 show that the decrease in the activity of encapsulated growth factors is much lower than free growth factor in the aqueous medium which means that the



**Fig. 2.** Confocal microscopy micrographs (central section). The distribution of FITC-BSA within the microspheres after (a) 30 minutes, (b) 1 day and (c) 7 days.



**Fig. 3.** Decrease in the activity of both growth factors in PBS, very fast decrease is shown at room temperature for both growth factors. At 5° slower decrease was remarked but is still fast comparing with culture medium.

polymeric membrane enhances the stability of the growth factor. It is interesting here to investigate the temperature effect and the microspheres size and permeability on the encapsulated growth factor stability. Figure 5 shows, as expected, that loading decreases more rapidly at higher temperature. It is interesting to notice also that the shelf life of big microspheres loaded with FGF-2 is somewhat shorter than smaller microspheres.

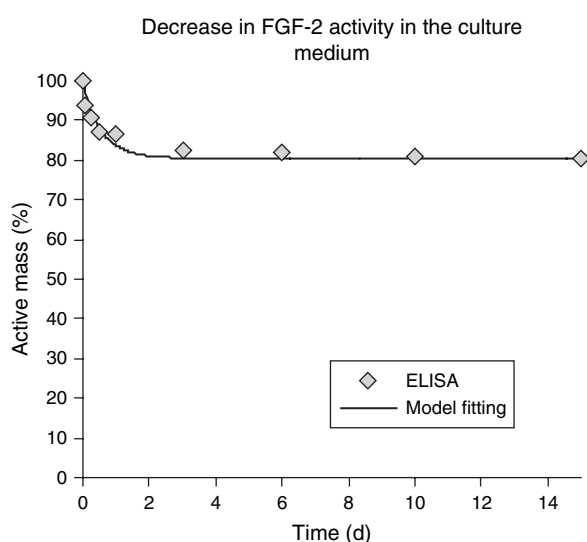
Figure 6 shows that after 21 days, the microspheres shelf life is proportional to the w/o ratio in the internal phase (for TGFβ1 a slight effect of internal ratio was found after only 6 days). A more compact microsphere (lower w/o internal ratio) leads to an improved protection of the growth factor.

#### 4.8. Microspheres Degradation

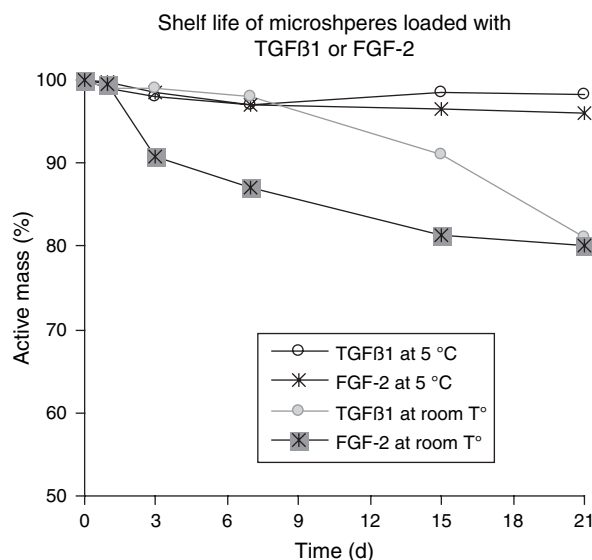
From the microspheres SEM pictures (Fig. 7), it can be seen that the spherical shape of the microspheres is deformed with time and surface irregularities appear with some pores on the surface and even some exploded microspheres can be detected.

Microspheres surface morphology observed by AFM microscopy after 30 days in water reveals a soft microsphere with a rough and irregular surface and confirms the loss of surface smoothness (Fig. 8).

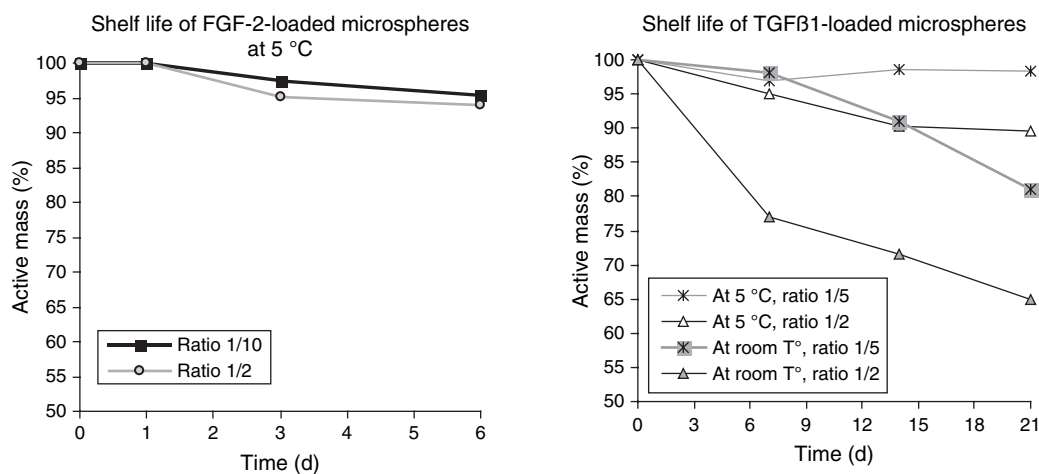
SEC measurements (Fig. 9) show a slight decrease in the PLGA molecular weight during the contact with water.



**Fig. 4.** Decrease of the activity of FGF-2 in culture medium at room temperature. The protein degradation rate coefficient represents the aggression of the medium. This coefficient was fitted using the ELISA data to be  $k = 6.6e - 4 \text{ ng}^{-1} \cdot \text{h}^{-1}$ .



**Fig. 5.** Shelf life of FGF-2-loaded microspheres as a function of the particle size and temperature (w/o internal ratio = 1/5). The loading decreases more rapidly at higher temperature. The shelf life of big microspheres loaded with FGF-2 is shorter than smaller microspheres.



**Fig. 6.** Shelf life of small microspheres as a function of the internal w/o ratio for both growth factors. A very slight effect was found in the first 6 days as seen for TGF $\beta$ 1. A significant effect of internal ratio was found after 21 days as seen for FGF-2.

This decrease is (partly) responsible of the polymeric matrix erosion and should affect the growth factors diffusion in the matrix and therefore the release rate.

#### 4.9. *In Vitro* Release Study

The release kinetics showed the existence of a phase of rapid release during the first 24 hours in which about 30–56% of the drug is released (Figs. 10–11). This phenomenon is described in the literature as the burst effect and can be beneficial in order to ensure the therapeutic dose (see for instance Ref. [4]). This phenomenon can be due to the non homogeneity of the matrix that contains big and small cavities. Bigger cavities might be formed preferably close to the microspheres surface due to violent solvent extraction. Diffusion out of big cavities is rapid and therefore comes quickly to end while diffusion through very small cavities is lower and continue for a longer period of time.

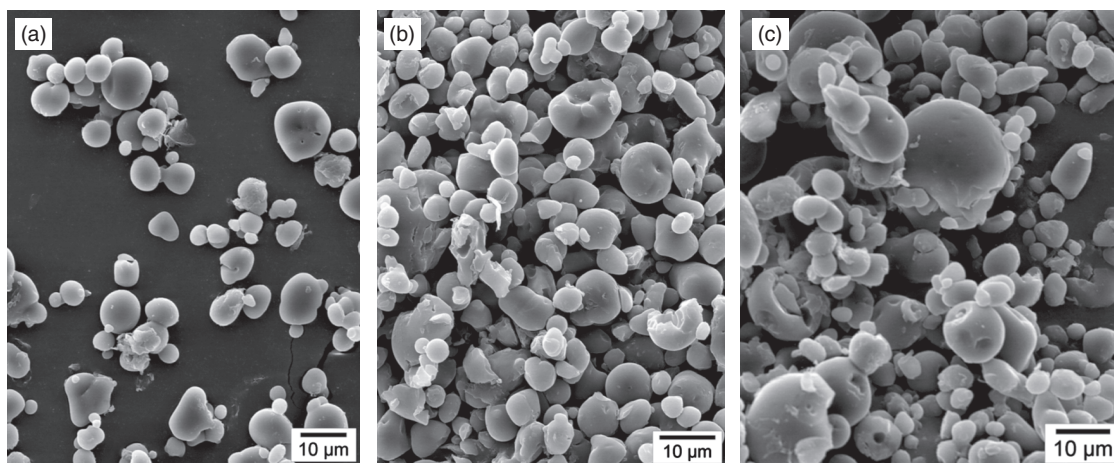
Figure 10 compares the amount of FGF-2 released with time as a function of the microspheres size. It can be seen

that almost the same amount is released from both small and big microspheres. Since small microspheres have a higher contact surface area with the release medium, then it can be concluded that the diffusion coefficient of small microspheres is lower than bigger ones.

The effect of the internal w/o ratio on the diffusion rate can be observed on Figure 10. It can be seen that the released amount of drug is proportional to the internal w/o ratio. Since the internal w/o ratio had no effect on the particle size as reported previously by our team,<sup>15</sup> then it can be concluded that a higher diffusion coefficient is obtained for higher internal w/o ratio.

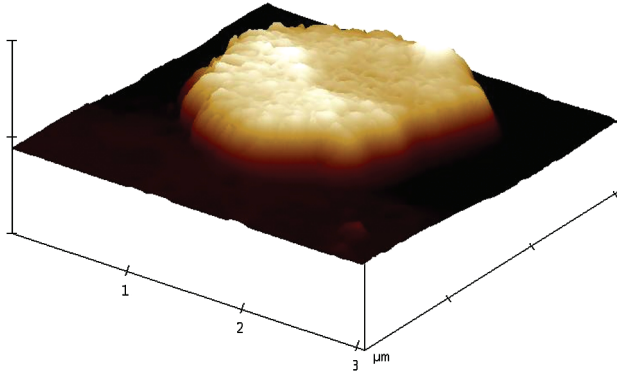
#### 4.10. Estimation of the Diffusion Coefficient

The second Fick's law of diffusion<sup>32</sup> was used to estimate the diffusion coefficient. In spherical particles, an analytical solution of this law can be derived assuming homogeneous dispersion of the drug in the sphere, constant diffusion coefficient on the particle radius ( $r$ ) perfect



**Fig. 7.** SEM pictures showing the degradation of the microsphere after (a) 7 days, (b) 14 days and (c) 30 days of suspension in PBS.





**Fig. 8.** Microspheres surface morphology by AFM after 30 days in water.

sink conditions and a drug loading that is lower than the solubility of the drug inside the polymer matrix:

$$\frac{M_t}{M_\infty} = 1 - \frac{6}{\pi^2} \sum_{n=1}^{\infty} \frac{1}{n^2} \exp\left(\frac{-Dn^2\pi^2 t}{r^2}\right) \quad (1)$$

where  $M_t$  and  $M_\infty$  are the cumulative absolute amounts of the drug released at time  $t$  and at infinite time respectively,  $r(\mu\text{m})$  is the average microspheres size and  $D(\mu\text{m}^2/\text{s})$  is the apparent diffusion coefficient.

In order to take in account the stability of released growth factors in the culture medium, we consider data given in Figure 4. It can be seen that the decrease in the growth factor activity is rapid at the beginning but almost stops after few days. Protein degradation is a complex domain that involves different chemical and physical pathways. The decrease in the protein activity in the release medium due to interactions with this medium (depending on the type of solvent, temperature, pH, presence of protective excipients...) is represented by superficial amount of the protein ( $X$ ). The reduction in the growth factors

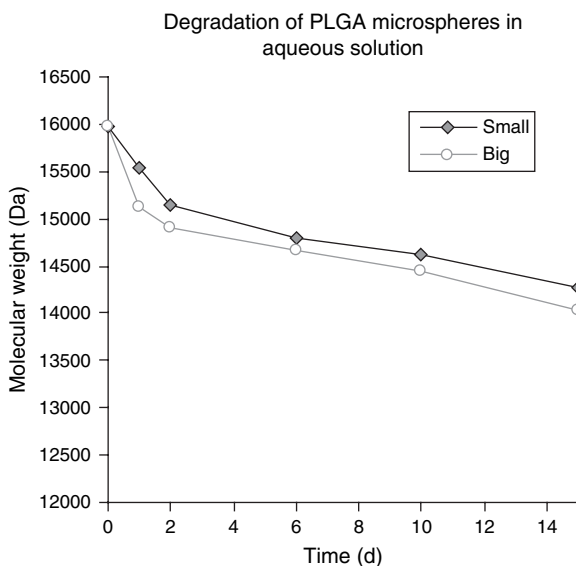
activity in water and in the culture medium can mathematically be represented by the following system:

$$\begin{cases} \frac{dM_t}{dt} = \underbrace{\Delta M_{t,\text{released}}}_{\text{Input due to release}} - \underbrace{k M_t X}_{\text{Output due to degradation}} \\ \frac{dX}{dt} = -k M_t X \end{cases} \quad (2)$$

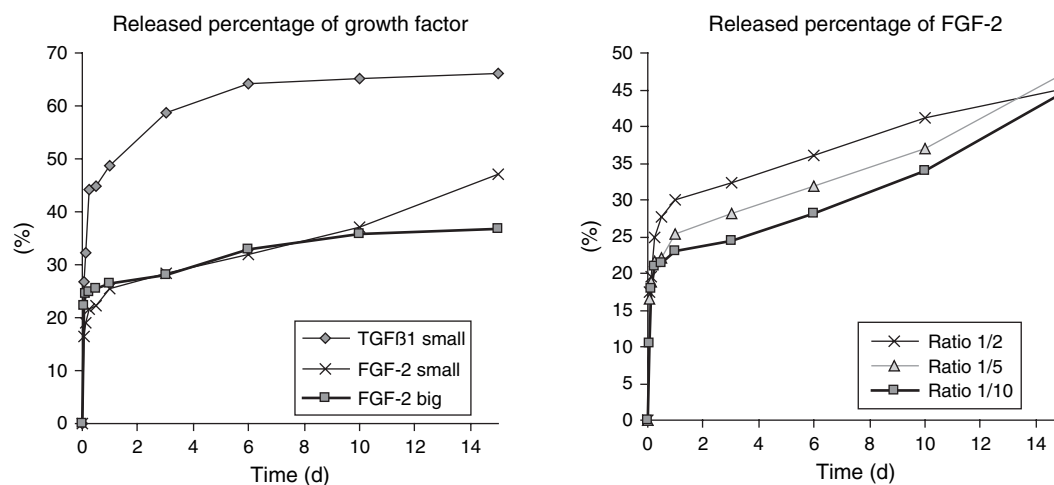
Where  $M_t$  is the protein mass and  $k$  is the protein degradation rate coefficient (Fig. 4). This unique coefficient represents the aggression of the considered medium. This coefficient was fitted using the ELISA data to be  $k = 6.6e-4 \text{ ng}^{-1} \cdot \text{h}^{-1}$ . In this model, the initial value of  $X(\text{ng})$  is the amount of  $M_t$  to be denatured in the considered medium. When  $X$  is totally consumed,  $M_t$  becomes stable. In Eq. (2),  $\Delta M_{t,\text{released}}$  = the released amount of drug during the sampling period.

In order to estimate the diffusion coefficient, one has to take in consideration the diffusion rate of the drug and the reduction in its activity simultaneously (Eqs. (1) and (2)). An optimization example is shown on Figure 11. The figure shows the released and residual amounts of growth factor obtained with the optimized diffusion coefficient. It can be seen when comparing the curves referred to as 'Released' and 'Residual' that an important amount of the drug is degraded during the release study. In these curves, a time-constant diffusion coefficient is considered (Table III) which assumes that the matrix porosity is homogeneous. However, the occurrence of a burst effect reveals some heterogeneity in the matrix (presence of small and big cavities) which leads to a variation in  $D$  with time. Therefore, the same optimization method explained above was applied by authorizing  $D$  to vary with time. Actually, optimization is done over 3 data measurements at a time. Then, the optimization recedes by ignoring the oldest data point and adding a new point at the right hand side of the figure and so on. The released and residual curves obtained by the receding horizon optimization are shown on Figure 11 and referred to as 'adaptive'. It can be seen that a more precise fitting is obtained in this case. The time-varying estimated diffusion coefficients as obtained by the adaptive method are shown on Figure 11. It can be seen that  $D$  decreases with time. It is important to remind that the molecular weight of polymer slightly decreased with time which could increase the diffusion rate, but this was not the case, therefore it was not necessary to incorporate degradation of the polymer molecular weight in the model.

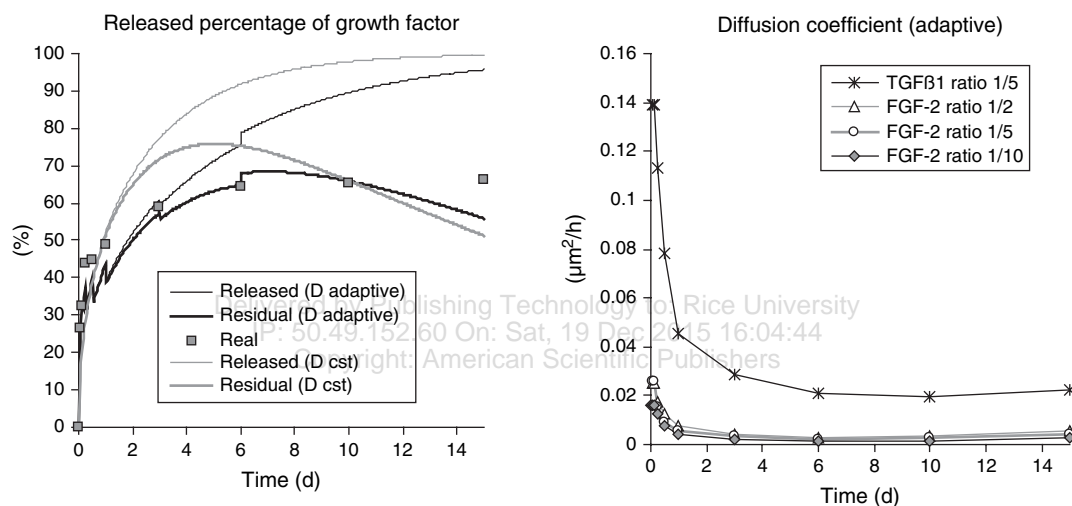
The time-constant diffusion coefficients estimated in these experiments are shown in Table III. The table confirms our expectations regarding the size effect and the internal w/o ratio on the diffusion coefficient. It can be seen that the diffusion coefficient increases with increasing the internal w/o ratio which increases the microspheres



**Fig. 9.** Degradation of the polymer matrix measured by SEC.



**Fig. 10.** Release profile of FGF-2- and TGF $\beta$ 1-charged microspheres as a function of the microspheres size and w/o ratio in the internal phase.



**Fig. 11.** At left, identification of  $D$  in experiment 2b. At right: Estimation of  $D$  by using receding horizon optimization for small microspheres ( $D$  of experiment 4a, bigger microspheres, goes from 6 to  $0.3 \text{ ng}^2/\text{h}$ ). Adaptive refers to a receding horizon optimization (varying  $D$ ), otherwise  $D$  is constant.

porosity. It can also be seen that the diffusion coefficient of small particles is much lower than that of bigger ones. Finally, the diffusion coefficient of TGF $\beta$ 1 (25 KDa) (microspheres size  $\approx 137 \mu\text{m}$ ) is higher than that of FGF-2 (17 KDa) (microspheres size  $\approx 93 \mu\text{m}$ ). The release rate difference should mainly be due to differences in the molecular nature between these growth factors, since differences in the size are negligible. When comparing to the diffusion coefficient of bovine serum albumin (BSA) (fraction

V, 60 kDa) estimated previously,<sup>15</sup> it could be seen that the diffusion coefficient was 1000 times higher for BSA than FGF-2 for both big and small particles.

## 5. DISCUSSION

Spherical microspheres with a smooth surface were formed by the proposed method. Dependence of the microsphere size on the stirring energy was confirmed. Concerning the microspheres biocompatibility, it was evaluated by measuring the residual solvent and surfactant concentrations. Indeed, DCM is an organic solvent that can be dangerous for humans when inhaled at a high concentration and was found to cause cancer in humans exposed to vapours in the workplace.<sup>24</sup> Studies of Serota et al. recommends that exposure of children to DCM be limited to less than 5 mg/L of drinking water for 1 day.<sup>24,25</sup> In the produced microspheres, DCM concentration was about

**Table III.** Estimation of the diffusion coefficient.

Experimental conditions (a stands for FGF-2 and b for TGF $\beta$ 1)	Average diffusion coefficient ( $\mu\text{m}^2/\text{h}$ )
1a (Small microspheres, ratio 1/2)	0.0053
2a (Small microspheres, ratio 1/5)	0.0039
3a (Small microspheres, ratio 1/10)	0.0027
4a (Big microspheres, ratio 1/5)	0.5469
2b (Small microspheres, ratio 1/5)	0.0424

3 ppm per mg of microspheres, which allows the administration of several milligrams of microspheres without any risk. The PVA concentration was about 0.5% by weight. This concentration is also lower than the potential toxic dose.<sup>26</sup> The obtained microspheres loading (2.9 ng/mg for FGF-2 and 1 ng/mg for TGF $\beta$ 1) is appropriate for local applications since the therapeutic doses of growth factors are usually between 10 and 20 mg of microspheres.<sup>27–31</sup> The produced microspheres are therefore adequate for human use.

It can be confirmed that the polymeric membrane enhances the stability of the growth factor. The shelf life of the growth factor-loaded microspheres is much higher than solutions of these growth factors. At ambient temperature, more than 80% of growth factor in water solution was lost in 15 days. Only 20% of encapsulated growth factor was lost after 15 days at ambient temperature. In the other side, storing microspheres in freezer keep them entirely actives.

Since the produced microspheres are designated for dentin-pulp complex regeneration, the release study was done in a culture medium that is usually used for *in vitro* tests on cultured cells. Even though confocal microscopic images show a homogeneous distribution of the FITC–BSA in the microspheres and a gradual diffusion of FITC–BSA through the microsphere with time, a burst effect took place in this system which should be due to the presence of different sizes of cavities in the microspheres as previously shown by SEM.<sup>15</sup> The effect of alterations in the microspheres morphology and degradation of the polymer molecular weight on the protein diffusion coefficient can be supposed to be negligible during the considered period of time as supported by mathematical modelling and SEM and SEC analysis.

It is observed that the production of smaller microspheres has several beneficial effects. First of all, the encapsulation efficiency is higher in smaller microspheres. Second, the shelf life of small microspheres loaded with FGF-2 is somewhat longer than bigger microspheres. Third, the polymer molecular weight of microspheres suspended in aqueous solution decreases less rapidly in smaller microspheres even though their surface area is bigger. Fourth, the diffusion rate coefficient is lower in smaller microspheres. The explanation for these observations can be the following: during the formulation process, for small microspheres the polymeric precipitation is done more rapidly protecting thereby a higher amount of growth factor than bigger ones. It seems also that a higher compact polymer matrix is formed if polymeric precipitation is rapid which prevents explosion of the primary cavities. By this way, the protein diffusion coefficient out of the microspheres is lower in smaller microspheres. Water diffusion into the microspheres should also be slower and therefore the degradation of the polymer molecular weight is slower

in smaller microspheres. Finally, the stability of the protein in smaller microspheres is enhanced due to the higher permeability ensured by the compact matrix.

## 6. CONCLUSIONS

FGF-2 and TGF $\beta$ 1-loaded microspheres were prepared, characterized and their release profile was examined. The developed method was found to be adapted to growth factors encapsulation and led to an encapsulation efficiency of about 35%. Microspheres' loading is adequate for therapeutic applications. The obtained microspheres have no potential toxicity regarding the PVA and solvent content. The microspheres enhance the stability of the growth factors and ensure controlled release. The activity of the encapsulated growth factor was conserved and the microspheres were found to be biocompatible and adapted for tissue engineering.

The internal morphology of the microspheres was found to be related to the precipitation time of the polymer. Smaller microspheres are supposed to precipitate faster due to their higher surface area. It was found that smaller microspheres have a higher compact matrix. Indeed, the drug stability is enhanced in smaller microspheres. The degradation rate of the polymer molecular weight of smaller microspheres is lower than bigger ones and the diffusion coefficient of smaller particles is much lower than bigger ones. Note that this last comment does not mean that diffusion rate out of smaller microspheres is lower than bigger ones since their surface area is higher. Added to all these advantages of smaller microspheres, their encapsulation efficiency is also higher than bigger ones. Therefore, fabrication of small microspheres is recommended to have optimized microspheres characterizations such as low release rate and high drug stability.

It is amazing to see that combination of some characterization and process modelling could give information about the internal morphology of the microspheres while small microspheres could not be easily cut into parts to be observed by microscopy as done for bigger microspheres.<sup>15</sup>

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