In-vitro interferon-α release from interferon-α and pegylated IFN-α loaded PLGA and PEG-PLGA nanoparticles

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Abstract

Aims: Interferon alpha-2a (IFN-α) controlled release of nanoparticles was investigated under in vitro conditions. **Materials & Methods**: IFN-α and pegylated IFN-α (PEG-IFN-α) were encapsulated by poly(lactic-co-glycolic acid) (PLGA) and pegylated PLGA (PEG-PLGA) copolymers using double emulsion solvent evaporation method. **Results**: The size of resulting four nanoparticles (IFN-PLGA, IFN-PEG-PLGA, PEG-IFN-PLGA and PEG-IFN-PEG-PLGA) was below 130 nm diameter. IFN-α encapsulation efficiency of the nanoparticles was between 78% and 91%. **Conclusions**: The *in-vitro* drug release studies conducted in phosphate buffered saline (PBS) and human plasma highlighted the role of incubation medium on the interferon release from the nanoparticles. The PEG-IFN-PEG-PLGA was the most promising nanoparticle among the four formulations because of its remarkably constant release in both PBS and plasma.

Keywords: interferon-α, nanoparticles, in-vitro release.

1. Introduction

Interferon alpha (IFN-α) is a cytokine produced by leukocytes upon exposure to viruses, mitogens, tumor cells or foreign cells. IFN-α shows immunmodulating, growth inhibitory, antiviral and antitumor activities [1]. IFN-α is widely used in the treatment of chronic hepatitis B, chronic hepatitis C, condylomata acuminata and several malignant neoplasms including AIDS-related Kaposi's sarcoma, hairy-cell leukaemia, chronic myeloid leukaemia, follicular lymphoma, cutaneous T-cell lymphoma, carcinoid tumours, melanoma, multiple myeloma, and renal cell carcinoma [2].

Whilst IFN-alpha has great potential as a potent anti-viral, anti-cancer and immunomodulatory agent, its clinical efficacy, however, is limited by some drawbacks. Interferon alpha is very sensitive to degradation and is also rapidly cleared from blood circulation by reticuloendothelial system (RES). In order to enhance the drug's therapeutic potential by prolonging its circulation time and also to reduce its side effects, a number of modifications to the molecule have been introduced or attempted. One such modification is the addition of polyethylene glycol ('pegylation') to the molecule to reduce reticuloendothelial cell-mediated clearance. This technique has, indeed, proven very effective in prolonging the drug's half life and reducing its unpleasant side effects.

A more recent innovative approach to achieve the desired goals of controlled drug release, long half life, targeted delivery and optimum toxicity profile is the technique of

'nanoencapsulation' using various nanoparticles. Nanoparticles such as liposomes and micelles have been widely used for nanoencapsulation. A number of liposome encapsulated anti-cancer and anti-microbial agents have entered routine clinical arena. Another class of promising nanoparticles for encapsulation are the FDA approved biocompatible and biodegradable polymers such as poly (D,L-lactic-co-glycolic acid) (PLGA), poly (D,L-lactic-acid) (PLA), poly (D,L-lactic-co-glycolic acid)-polyethylene glycol (PEG-PLGA) [3,4]. Micro/nanoencapsulation of interferon alpha has been studied by several research groups. Sanchez et al. [5] formulated PLGA micro- and nanoparticles containing IFN-α co-encapsulated with poloxamer and HSA by a double emulsion technique and PLGA/poloxamer blend microspheres containing IFN-α by oil-in-oil solvent extraction technique. Giri et al. [6] synthesised IFN-α loaded cationic PLGA nanoparticles with delipidated HBsAg adsorbed on the surface of nanoparticles. Besides PLGA, a number of other micro/nanocarriers such as poly-ε-caprolactone microspheres [7], lipid-based nanostructures [8] and lipid coated aquasomes [9] have also been investigated as human IFN-α delivery systems. However, it is well-known that serum proteins are easily adsorbed onto hydrophobic polymeric nanoparticles such as PLGA [10], and the so-called opsonisation results in the fast clearance of these nanoparticles by the macrophages of RES from the blood circulation. Biodegradable hydrophilic molecules, such as PEG can effectively inhibit such protein adsorption and reduce clearance by RES.

The aim of this work was to encapsulate IFN-α and pegylated IFN-α (PEG-IFN-α) by poly(lactic-co-glycolic acids) (PLGA) and poly(lactic-co-glycolic acid)-polyethylene glycol (PEG-PLGA) copolymers using double emulsion solvent evaporation method and to compare the in-vitro release of interferon from the resulting four distinct nanoparticles, namely IFN-α in poly(lactic-co-glycolic acids) (IFN-PLGA), PEG-IFN-α in poly(lactic-co-glycolic acids) (PEG-IFN-PLGA), IFN-α in poly(lactic-co-glycolic acid)-polyethylene glycol (IFN-PEG-PLGA) and PEG-IFN-α in poly(lactic-co-glycolic acid)-polyethylene glycol (PEG-IFN-PEG-PLGA). To our knowledge, the two types of pegylated IFN-α containing nanoparticles are of novel composition. Furthermore, we are of the opinion that the in vitro investigations performed in both phosphate buffered saline and human blood plasma in this study should become the standard method to evaluate IFN-α release in-vitro, since this approach has revealed important new aspects in IFN-α release study. It is also emphasized that there is a long development and improving work behind the results of the present study. Some of our previous and recent papers [10-15] contain the most important achievements during the

optimization of size and encapsulation efficiency of PLGA nanoparticles containing model proteins, while the properties of magnetic PLGA nanoparticles with the IFN-α active agent were initially improved in a PhD thesis [16]. In the present study we used all of the knowledge obtained during the almost 10-year development of PLGA nanoparticles to produce optimal interferon alpha containing nanoparticles.

2. Material and methods

2.1 Material

PLGA (50:50, M_w = 8000, Resomer® RG 502H) with free carboxyl end groups was supplied by Boehringer Ingelheim, Germany. PLGA-PEG (RESOMER Select 5050 DLG PEG 6000 (10 wt% PEG)) was obtained from Evonik, US. Dichloromethane (DCM) was purchased from Scharlab, Hungary. Polyvinyl alcohol (PVA, M_w = 30,000–70,000), and phosphate-buffered saline (PBS, pH 7.4) were products of Sigma-Aldrich, Germany. Poloxamer (Pluronic® F68, M_w = 8350) was from BASF, Germany. Roferon A® (human IFN α-2a) and Pegasys 180 (pegylated human IFN α-2a) were obtained from Roche, Switzerland. The ELISA kits were purchased from IBL International Gmbh, Germany. The human blood plasma was supplied by the Hungarian National Blood Transfusion Service, Hungary.

2.2 Preparation of IFN-alpha-loaded nanospheres

Nanoparticles were prepared by double emulsion solvent evaporation method [10]. Briefly, 50 mg PLGA or PEG-PLGA was dissolved in 5 ml DCM. 0.5 ml protein solution consisted of Roferon A® (theoretical IFN α-2a concentration: 66 μg/ml) diluted 20 times by PBS, or Pegasys 180 (theoretical IFN α-2a concentration: 360 μg/ml) diluted 100 times by PBS was added to the organic phase, and the two phases were emulsified for 30 s by a probe sonicator (Model W-220, Heat Systems-Ultrasonics) at a power of 70 W and frequency of 20 kHz. This emulsion was added to 10 ml 2 wt% aqueous PVA solution and sonicated as described before but for 60 s to obtain w/o/w double emulsion. The DCM was evaporated under continuous stirring (800 rpm) for 3 h using a magnetic stirrer (IKA RET B). After the evaporation of DCM, dispersed solid nanoparticles were obtained and stored at -20 ºC for further experimental analysis.

2.3 Determination of particle size and encapsulation efficiency

The size of the nanoparticles was analyzed by dynamic light scattering method using Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) at 25°C. The encapsulation efficiency was determined indirectly. I.e. 2ml suspension (nanoparticle concentration: 4.76 mg/ml) of IFN-α-loaded nanoparticles was centrifuged (Beckman Optima Max-E) for 25 min at 30 000 g, and the supernatant was removed for determining the non-encapsulated IFN-α concentration by ELISA. The encapsulation efficiency (Ee) was calculated from Eq.1.

$$Ee = \frac{m_{\max} - m_{nenc}}{m_{\max}} 100, \quad (1)$$

where m_{max} marked the initial IFN-α mass, which was aimed to be microencapsulated, while m_{nenc} denoted the non-encapsulated IFN-α mass that was determined by ELISA in the supernatant after the microencapsulation process.

2.4 In-vitro interferon release assay

For in-vitro release investigations the pellet was resuspended in 2ml human blood plasma or 2ml PBS each containing 0.03wt% sodium azide bactericide, then the suspension was pipetted into sterile 5 ml microtube. The suspension containing microtubes were incubated at 37 ºC, and shaked at 250 rpm in a G24 Environmental Incubator Shaker (New Brunswick Scientific, USA). At pre-determined intervals, 0.1 ml of each samples was ultracentrifuged (Beckman Optima Max-E) for 25 min at 30 000 g, and the supernatant was removed for analysis of IFN-α. The amounts of non-encapsulated and released IFN-α were determined using the ELISA kits.

3. Results and discussion

3.1 Size and encapsulation efficiency of nanoparticles

The mean diameter of the four nanoparticles ranged between 104 nm and 129 nm with polydispersity index (PDI) of 0.11-0.16 (Figure 1). The mean diameters of IFN-α in poly(lactic-co-glycolic acids) (IFN-PLGA), PEG-IFN-α in poly(lactic-co-glycolic acids) (PEG-IFN-PLGA), IFN-α in poly(lactic-co-glycolic acid)-polyethylene glycol (IFN-PEG-PLGA) and PEG-IFN-α in poly(lactic-co-glycolic acid)-polyethylene glycol (PEG-IFN-PEG-PLGA) were 129 nm, 118 nm, 119 nm and 104 nm, respectively.

Encapsulation efficiencies, which were indirectly investigated from the supernatants obtained by ultracentrifugation, were as high as 90.6%, 87.9%, 87.3% and 78.2% for IFN-PLGA, PEG-IFN-PLGA, IFN-PEG-PLGA and PEG-IFN-PEG-PLGA respectively.

3.2 In-vitro studies

Though *in-vivo* release studies provide more reliable profile of the sustained delivery systems, *in-vitro* tests can also give useful information about their stability in aqueous medium. Another advantage of *in-vitro* release studies is that they are relatively inexpensive to perform. In recent studies carried out with micro- or nanoparticles designed for parenteral sustained delivery of IFN-α [5,7,9], the *in-vitro* release experiments were performed exclusively in phosphate buffered saline (PBS). Because blood proteins, especially albumin, have an important protective role towards the interferon molecule, we investigated the *in-vitro* release profiles both in human blood plasma and PBS. In preliminary examinations (data not shown), we found that poloxamer coating adsorbed onto PLGA nanoparticles (for method description see [10]) containing IFN-α or PEG-IFN-α caused dramatic decrease (50-80%) in IFN-α release. It is not clear whether this observation is the result of poloxamer mediated degradation of the IFN molecule or is due to poloxamer mediated inhibition of IFN release from polymer nanoparticles.

Figure 2a shows the recovery values of IFN-α and PEG-IFN-α during a prolonged incubation of these formulations in normal human plasma (control experiment) and Figures 2b and 2c chart the changes in interferon concentrations in normal human plasma and PBS respectively, during prolonged *in-vitro* incubation of the four different nanoparticles containing IFN-α or PEG-IFN-α. Surprisingly, both IFN-α preparations (unpegylated and pegylated) demonstrated high stability in plasma, preserving about 70% of its baseline level after 16 days. The baseline concentration values (one hour post incubation) in the incubation medium (plasma) were remarkably similar for IFN-PLGA, PEG-IFN-PLGA and IFN-PEG-PLGA (range 20.6-25.2 ng/l) (Figure 2b). However, the baseline concentration of IFN in incubation medium containing PEG-IFN-PEG-PLGA was less than half (9.0 ng/l) that of the other three nanoparticles. All four nanoparticles showed remarkable sustained release properties. The IFN release from both IFN-α and PEG-IFN-α containing PLGA nanoparticles was 90% of the baseline level at the end of the 16-day release study. IFN-α encapsulated in PEG-PLGA showed consistently lower concentrations (60-70% of baseline) compared to IFN-PLGA and PEG-IFN-PLGA nanoparticles. In contrast, remarkably, pegylated interferon encapsulated in pegylated PLGA (PEG-IFN-PEG-PLGA) produced sustained release of the encapsulated drug at levels up to 117% of the baseline concentration.

As expected, incubation medium exerted significant impact on the interferon release from various nanoparticles. The interferon release from the four nanoparticles incubated in plasma was significantly different to that in PBS. But there were few similarities as well (Figures 2b & 2c). With the exception of pegylated IFN-α encapsulated in pegylated PLGA (PEG-IFN-PEG-PLGA) which yielded consistently high IFN-α concentrations in excess of 80% of the baseline value in PBS, other three nanoparticles released variable and inconsistent levels of IFN-α which were significantly lower than the levels recorded in plasma. It is very striking that the baseline IFN-α concentrations in PBS with the three nanoparticles (IFN-PLGA, PEG-IFN-PLGA and PEG-IFN-PEG-PLGA) were almost one order of magnitude lower (2.5ng/l, 3.3ng/l and 2.7ng/l respectively) compared to that seen in plasma. The worst performer was IFN encapsulated in PEG-PLGA (IFN-PEG-PLGA) which yielded exceptionally low levels of the drug in PBS, as was in plasma. The baseline level was 0.7ng/l and the drug was not measurable after four days (Figure 2c).

4. Conclusions

Based on the results of our in-vitro experiments, we conclude that the nanoparticle of pegylated IFN-α encapsulated in pegylated PLGA polymer displays remarkable drug release characteristics that would make this formulation particularly suited for sustained interferon delivery. Hence, this formulation merits further evaluation as a potential drug carrier. PLGA nanoparticles containing IFN-α or pegylated IFN-α also show promise. The ability of these nanoparticles to release IFN-α in a sustained manner has been confirmed. However, their true pharmacokinetic properties including their 'retainability' in blood stream or in the targeted organ can be only verified by *in-vivo* studies. It is evident that plasma constituents stabilise IFN-α thus rendering the drug more stable in blood. However, the *in-vivo* pharmacokinetic properties of the above nanoparticles cannot be deduced from the results of this study because the experimental conditions did not contain either the active enzymes that degrade interferons in blood and tissues or, more importantly, the cells of reticuloendothelial system such as macrophages that remove bio-incompatible particulates from the blood. Hence, *in-vivo* animal studies are warranted to establish the precise pharmacokinetics of these nanoparticles. It must also be added that the surface of the nanoparticles may need to be modified to decrease their hydrophobicity. An attempt to achieve this goal by adsorbing poloxamer on to the polymer nanoparticles has proved ineffective. Finally, any future studies to evaluate the true potential

of the above nanoparticles must ideally include specific cell targeting using various recognition ligands such as cell specific antibodies.

Summary Points

Interferon alpha (IFN-α) and pegylated IFN-α were encapsulated by poly(lactic-co-glycolic acid) and pegylated poly(lactic-co-glycolic acid) copolymers by double emulsion solvent evaporation method.

The aim was to sustain the active agent release by means of the microencapsulation.

The size of the four nanoparticles was found to be between 104 nm and 129 nm.

Nanoparticles of this size may be suitable tools for potential intravenous drug delivery.

The incubation medium influenced significantly the interferon release.

The baseline released IFN-α amounts in PBS were generally approximately one order of magnitude lower compared to that seen in plasma.

Three of the four types of nanoparticles showed promising release feature during 16 days in blood plasma by assuring almost constant IFN-α level.

Pegylated IFN-α encapsulated by pegylated poly(lactic-co-glycolic acid) displayed the most beneficial drug release characteristics.

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*to our knowledge IFN-α was entrapped with PLGA nanoparticles by a double emulsion technique for the first time.

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** IFN-α co-encapsulation with superparamagnetic iron oxide nanoparticles was achieved by PLGA nanoparticles.

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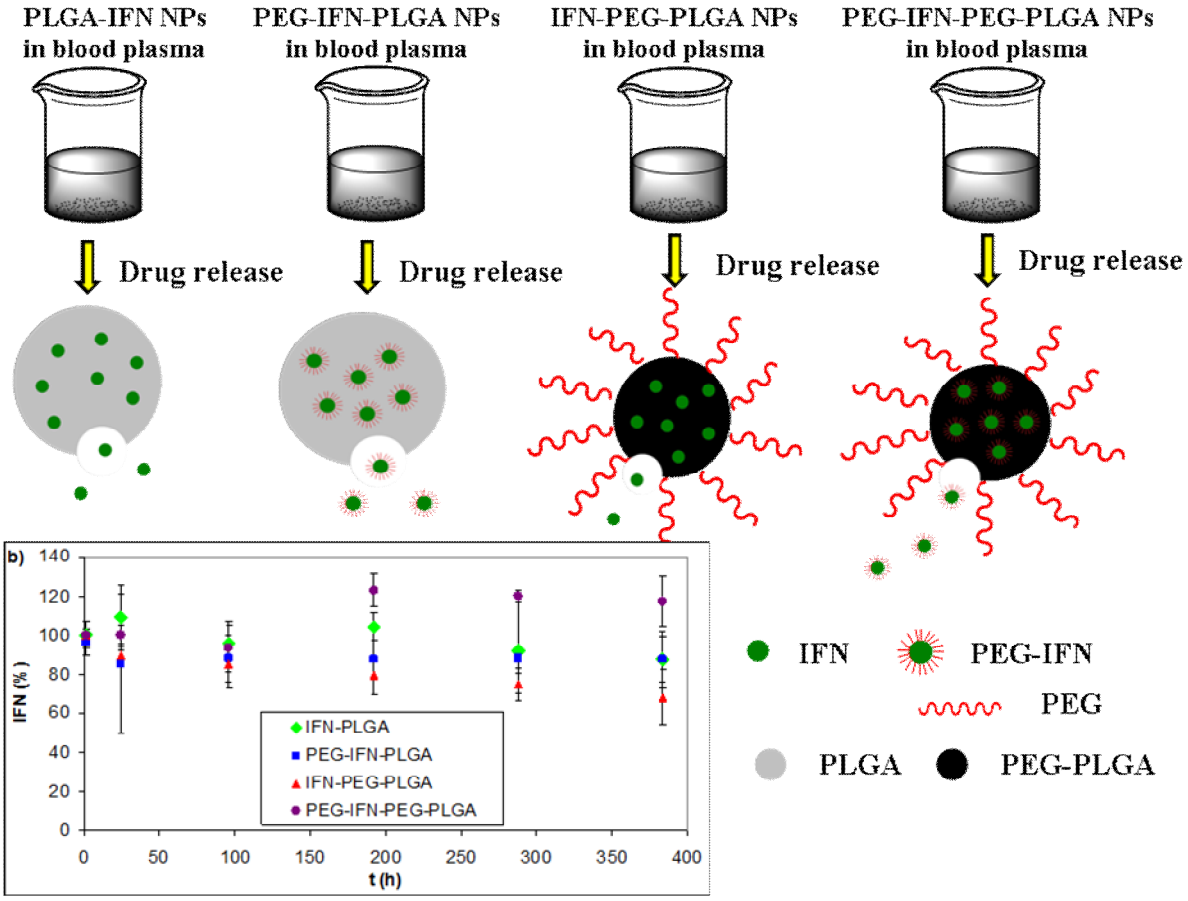
Graphical abstract



Captions of figures

Figure 1. Volume distribution of the nanoparticles: IFN-α in poly(lactic-co-glycolic acids) (IFN-PLGA), PEG-IFN-α in poly(lactic-co-glycolic acids) (PEG-IFN-PLGA), IFN-α in poly(lactic-co-glycolic acid)-polyethylene glycol (IFN-PEG-PLGA) and PEG-IFN-α in poly(lactic-co-glycolic acid)-polyethylene glycol (PEG-IFN-PEG-PLGA) were 129 nm, 118 nm, 119 nm and 104 nm, respectively.

Figure 2. (a) Stability of interferon-α (IFN) and pegylated interferon-α (PEG-IFN) in human blood plasma. (b) In-vitro release of interferon-α from nanoparticles during prolonged incubation in plasma and (c) in PBS. (abbreviations: IFN-PLGA - interferon-α in PLGA, IFN-PEG-PLGA - interferon-α in pegylated PLGA, PEG-IFN-PLGA - pegylated interferon-α in PLGA and PEG-IFN-PEG-PLGA - pegylated interferon-α in pegylated PLGA).

Fig1

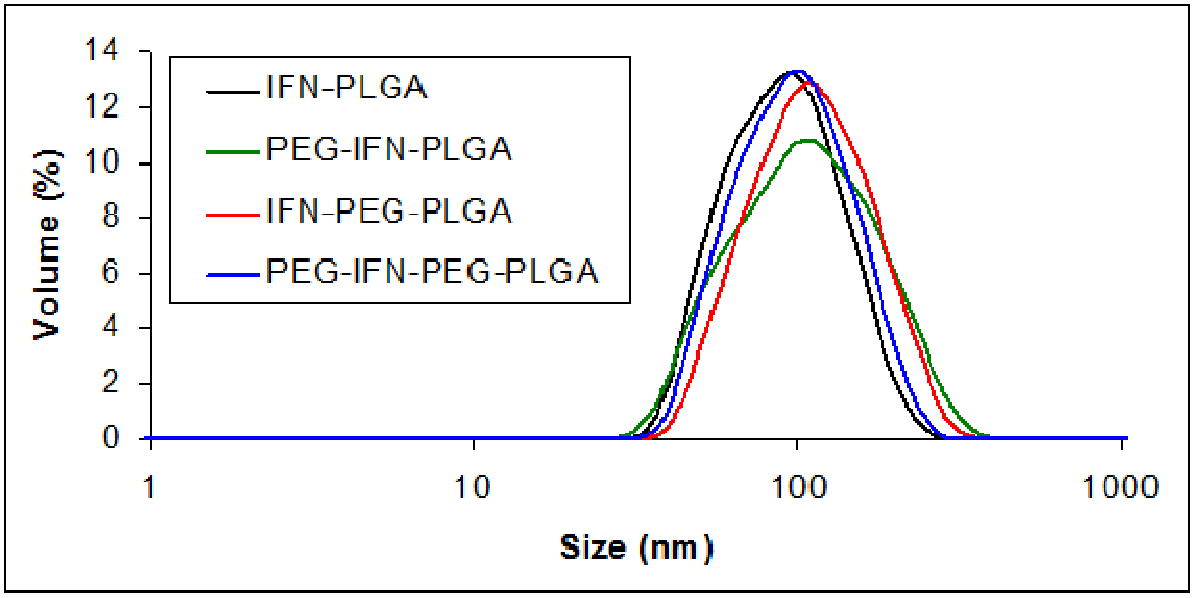

IFN-PLGA
PEG-IFN-PLGA
IFN-PEG-PLGA
PEG-IFN-PEG-PLGA
Volume (%)
Size (nm)
0
2
4
6
8
10
12
14
1
10
100
1000

Fig2a

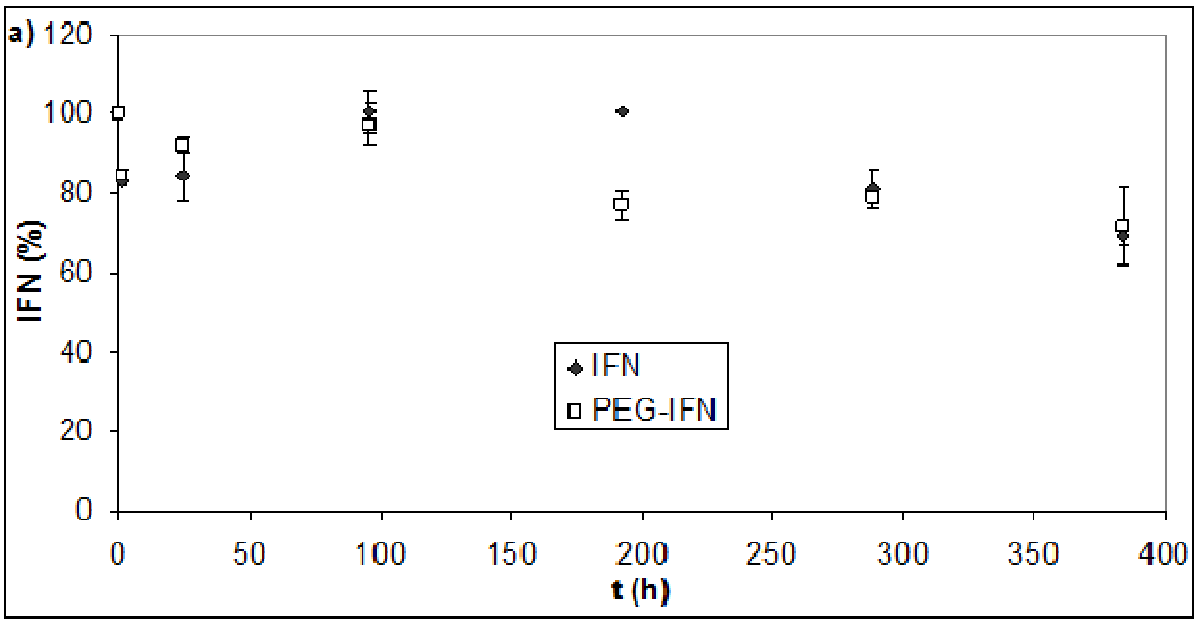

a)
IFN (%)
t (h)
IFN
PEG-IFN
0
20
40
60
80
100
120
0
50
100
150
200
250
300
350
400

Fig2b

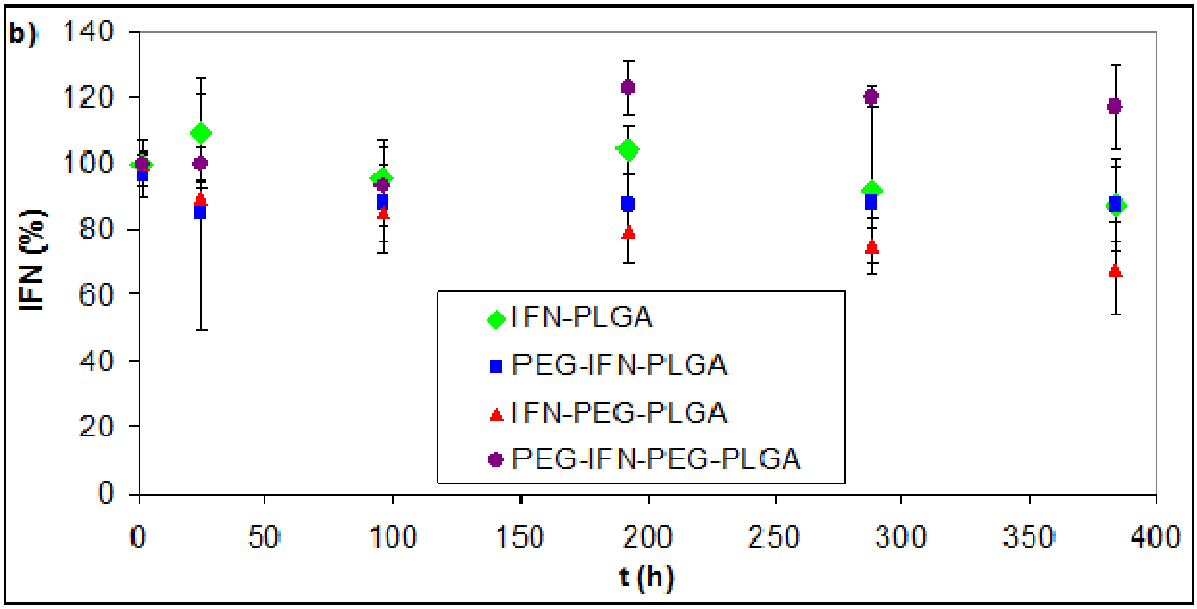
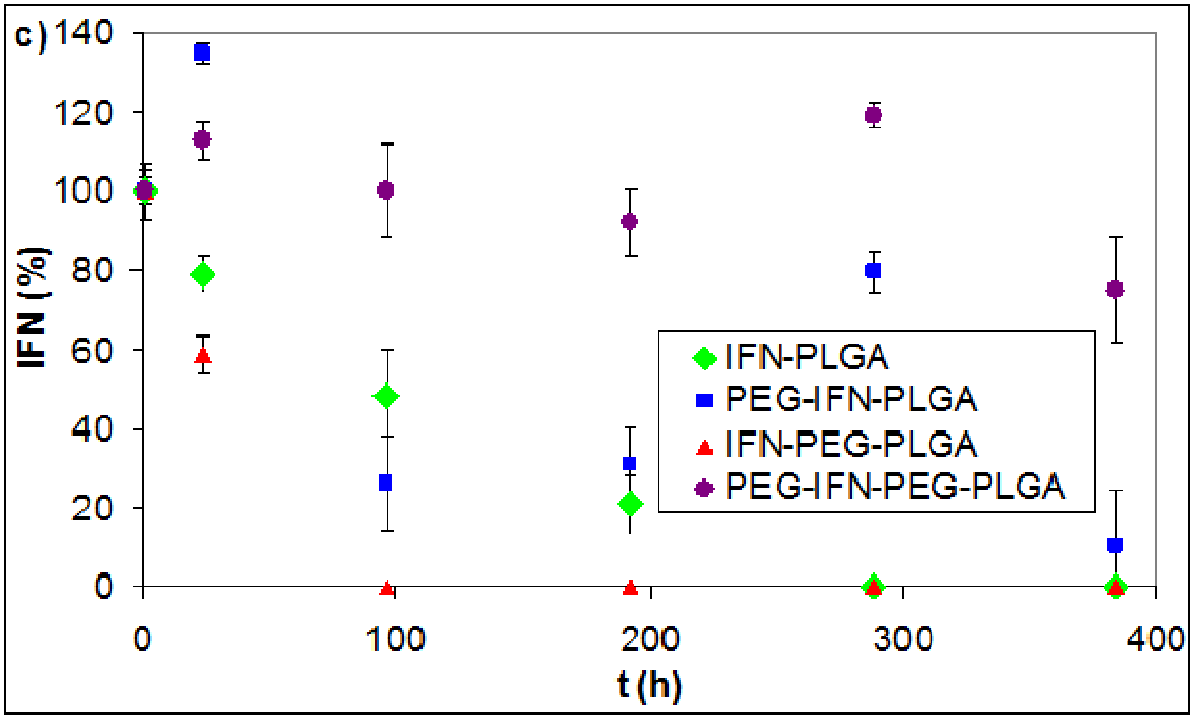

b)
IFN (%)
t (h)
IFN-PLGA
PEG-IFN-PLGA
IFN-PEG-PLGA
PEG-IFN-PEG-PLGA

Fig2c


c)
IFN (%)
t (h)
IFN-PLGA
PEG-IFN-PLGA
IFN-PEG-PLGA
PEG-IFN-PEG-PLGA