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Preparation and *in vitro* evaluation of carrier erythrocytes for RES-targeted delivery of interferon-alpha 2b

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Abstract

Carrier erythrocytes is one of the most promising systemic drug delivery systems investigated in recent decades. In this study, human erythrocytes have been loaded with interferon-α 2b (IFN) with the aim to benefit the reticuloendothelial system (RES) targeting potential of the carrier cells. Hypotonic preswelling method was used for drug loading in erythrocytes and the entire loading procedure was evaluated and validated. The loaded amount, entrapment efficiency and cell recovery of the loading procedure were 2906.33 ± 588.35 IU/0.1 ml, $14.53 \pm 2.94\%$, and $83.61 \pm 0.49\%$, respectively, all being practically feasible. The carrier erythrocytes were characterized *in vitro* in terms of their drug release kinetics, hematological indices, particle size distribution, SEM analysis, and osmotic and turbulence fragility. IFN release from carrier cells was a relatively rapid process in comparison to the cell lysis kinetics, which is unusual considering the whole body of data published on this delivery system and other protein drugs, so far. All the tested *in vitro* characteristics showed significant, sometimes notable changes upon drug loading procedure, both with and without the drug.

Keywords: Interferon; Interferon-α 2b; Carrier erythrocytes; Hypotonic preswelling

1. Introduction

Cellular carriers, including erythrocytes, leukocytes, platelets, islets, hepatocytes, and fibroblasts all have been exploited as potential carriers for bioactive substances, especially biopharmaceuticals, in recent decades (Banker and Rhodes, 2002; Hamidi and Tajerzadeh, 2003).

Erythrocytes as the most readily available and abundant cells in the body, have been studied extensively for their potential application as drug delivery carriers. The biodegradability, biocompatibility and considerable circulation life-span of the autologous carrier erythrocytes allow them to serve as prolonged release intravenous reservoirs for various drugs. On the other hand, as the reticuloendothelial system (RES) is the site of destruction for abnormal or aged erythrocytes, they can be used as drug targeting carriers to RES organs (Hamidi and Tajerzadeh, 2003).

Today, interferon-alpha (IFN-α), due to its antiviral (Baron et al., 1991; Basler and Garcia-Sastre, 2002), antitumor (Gresser and Belardelli, 2002; Kirkwood, 2002), and immunomodulatory (Kadowaki et al., 2000) effects, is the most widely used cytokine in clinic. IFN-α is used in over 40 countries for the treatment of more than 14 types of cancer, including some hematological malignancies and certain solid tumors as well as chronic hepatitis B and C (Novak, 2005). However, in spite of diverse indications of IFN as a leading biopharmaceutical, there are some inherent limitations regarding their wide application in clinical settings, including: short half-life (Al-Hasso, 2001; Novak, 2005) and serious adverse reactions (Novak, 2005) especially with high doses of IFN. Therefore, development of more efficient and safe IFN preparations is of special attention to reach a better efficacy as well as a higher degree of compliance among other clinical implications. Various novel drug delivery systems (NDDSs) (Kajihara et al., 2001; Sanchez et al., 2003; Pedder, 2003; Vyas et al., 2006) are among the most widely used approaches attempted to achieve long term and/or site-specific delivery of this therapeutic protein.

In this study, the human erythrocytes have been loaded by interferon-alpha 2b (IFN-α 2b) using a relatively destructive

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osmosis-based method and the *in vitro* characteristics of the prepared cellular carriers have been evaluated. As we believe that the use of a RES-targeted cellular carrier system using the autologous cells will direct a high dose of IFN to the RES in a short time period, thereby potentially augmenting the biological response using a relatively low drug dose, while having no non-specific carrier-associated effects, as seen with some polymeric systems, we hope this work will be a starting point for some beneficial studies on IFN delivery systems.

2. Materials and methods

2.1. Materials

IFN-α 2b was kindly donated by Pouyesh Darou Pharmaceutical Co., Tehran, Iran. ELISA kit for IFN-α assay was purchased from Biosource, USA (Lot. No. 2697A). All other chemicals and solvents were from analytical purity grade, purchased locally and used without further purification.

2.2. Drug assay

Considering the need for a highly sensitive method for *in vitro* evaluations of the carrier cells, we used a validated and commercialized enzyme-linked immunosorbent assay (ELISA) method (Biosource, USA) for this purpose. IFN-α 2b assay was performed using Human Interferon Alpha ELISA Kit (a sandwich immunoassay) according to its extended range (0–1250 IU/ml) protocol. Total sample preparation time was 195 min according to kit package instruction. To prepare samples for drug assay, 1 ml of washed loaded erythrocytes was diluted with 1 ml of distilled water (1:1) to produce IFN containing hemolysate for assay. Then, considering upper limit of extended range (*i.e.*, 1250 IU/ml), hemolysates were diluted before placing in wells. For drug assay in supernatant samples in release experiments or the wash solutions in loading procedure, there was no need for 1:1 dilution with distilled water and, after needed dilution, the assay protocol was performed.

2.3. Preparation of human erythrocytes

Blood samples were withdrawn by venipuncture from healthy volunteers aged from 20 to 35 years using 19-G hypodermic syringes and transferred to pre-heparinized polypropylene test tubes. After centrifuging at $1000 \times g$ for 10 min, the plasma and buffy coat were separated by aspiration, and the remaining packed erythrocytes were washed three times using phosphate-buffered saline (PBS; 150 mmol/l NaCl and 5 mmol/l K_2HPO_4, pH 7.4).

2.4. Encapsulation of IFN-α 2b in human erythrocytes

A hypotonic preswelling method described and validated by Tajerzadeh and Hamidi (2000) was used for loading the human erythrocytes with IFN-α 2b. For this purpose, 1 ml of washed packed erythrocytes was transferred gently to a polypropylene test tube, 4 ml of a hypotonic PBS with osmolarity of 0.67 that of the eutonic solution was added and the resulting cell suspension was mixed gently by 10-times inversion. The swollen cells were, then, separated by centrifuging at $1000 \times g$ for 10 min and the supernatant was discarded. A 200 μl aliquot of a hemolysate, prepared by diluting another portion of erythrocytes with distilled water (1:1), was added gently onto the remaining swollen cells, and then, 250 μl of an aqueous solution of IFN-α 2b (200,000 IU/ml) was gently added onto the cell suspension, and the resulting mixture was inverted gently several times and centrifuged at $1000 \times g$ for 5 min. The hemolysate layer serves as a osmotic shock barrier to cells and also as a reservoir of cell constituents preventing their loss during drug addition steps. Addition of IFN-α 2b solution, mixing, and centrifuging were repeated successively three more times to achieve the lysis point of the cells. This point was detectable by a sudden increase in transparency of the cell suspension and the disappearance of the distinct boundary between cells and supernatant on centrifuging. At this point, the erythrocytes were resealed by the rapid addition of 100 μl of hypertonic PBS with an osmolarity of 10 times of the eutonic solution, followed by gentle mixing of the suspension by several inversions. Finally, the resulting mixture was incubated at 37 °C for 30 min to reanneal the resealed cells. The carrier erythrocytes obtained by this manner were washed three times using 10 ml aliquots of PBS to wash out the unentrapped IFN-α 2b and the released hemoglobin and other cell constituents during the loading process.

In some experiments, the sham-encapsulated erythrocytes were needed, which were prepared as described except for the IFN-α 2b aqueous solution replaced by distilled water during the procedure.

2.5. Loading parameters

To evaluate the final erythrocyte carriers, three indices were defined as loading parameters:

- *Loaded amount*: The amount of IFN-α 2b entrapped in 0.1 ml (arbitary volume) of the final packed erythrocytes.
- *Efficiency of entrapment*: The percentage ratio of the loaded amount of IFN-α 2b to the amount added during the entire loading process.
- *Cell recovery*: The percentage ratio of the hematocrit value of the final loaded cells to that of the initial packed cells, both measured using equal suspension volumes.

2.6. Methodological tests

2.6.1. Incubation of intact erythrocytes with isotonic interferon-α 2b solution

To investigate the possible uptake and/or degradation of IFN-α 2b by intact human erythrocytes irrespective of loading condition, 0.5 ml of washed packed erythrocytes was incubated at 37 °C with 0.5 ml of IFN-α 2b solutions in PBS with a concentration of 200,000 IU/ml (the same concentration as used in loading procedure). At 15, 30, and 60 min, equal volumes of the cell suspension were harvested, centrifuged at $1000 \times g$ for 5 min, and the concentrations of remaining IFN-α 2b in the

supernatant were determined using the ELISA assay method. In addition, IFN-α 2b concentration in the cellular fraction was also determined at each time point after cell lysis by 1:1 dilution with distilled water.

2.6.2. Effect of concentration of IFN-α 2b solution

The entrapment procedure was performed using aqueous solutions of IFN-α 2b with concentrations of 10,000, 20,000, 50,000, 100,000, 200,000, 500,000 and 1,000,000 IU/ml, and the loading parameters were determined in each case.

2.6.3. Mechanism of entrapment

To elucidate the possible mechanism of entrapment, an entrapment procedure was performed, as described, and the concentrations of IFN-α 2b in each of three final washing solutions as well as in final packed cells were determined. Then, the total amount of washed out (unentrapped) protein was calculated by considering the total volumes of discarded solutions. On the other hand, the total amount of entrapped IFN-α 2b was determined using the loaded amount multiplied by the cell recovery of the method. Finally taking the volume fraction of cells in whole suspension at the lysis point, the mechanistic behavior of erythrocytes in the IFN-α 2b loading was exploited.

2.7. Process validation tests

To validate the entrapment process, the following tests were carried out.

2.7.1. Intra-subject variations

Three blood samples were collected from a healthy volunteer and the loading procedure was carried out on each sample separately. The loading parameters for each sample as well as the corresponding coefficients of variations (CV%) were determined.

2.7.2. Inter-subject and sex-related variations

Blood samples were collected from six healthy volunteers (three male and three female subjects), and the loading procedure was carried out in each case. The loading parameters for each of the subjects as well as the respective CV% were determined.

2.7.3. Recovery

The measured entrapped, unentrapped, and total amount of IFN-α 2b recovered after completion of the entrapment procedure were compared with those calculated by considering the total added IFN-α 2b amount and the volume fraction of cells and supernatant at the lysis point.

2.8. In vitro characterization of IFN-α 2b-loaded erythrocytes

2.8.1. IFN and hemoglobin release

To exploit the release kinetics of IFN-α 2b as well as hemoglobin (an indicator of cell lysis) from carrier erythrocytes, 0.5 ml of packed IFN-α 2b-loaded cells was diluted to 5 ml using Ringer solution containing 0.01% sodium azide (NaN_3) as an antimicrobial preservative for erythrocytes, and the suspension was mixed thoroughly by several gentle inversions. Then, the mixture was divided into ten 0.5-ml portions in 1.5 ml polypropylene microtubes. The samples were rotated vertically (15 rpm) while incubated at 37 °C using a vertically shaking incubator designed and assembled in-house. At the beginning of the test and also at 0.25, 0.5, 1, 2, 4, 8, 24, and 48 h intervals, one of the aliquots was harvested and after centrifuging at $1000 \times g$ for 5 min, 100 μl of the supernatants were separated for IFN-α 2b assay. In addition, the absorbance of a 0.3 ml portion of the supernatant was determined at 540 nm using a UV/visible spectrophotometer (Cecil, Series 9000, UK) to monitor the hemoglobin release. These experiments were carried out in triplicate and the percent of IFN-α 2b and hemoglobin release were determined in reference to a completely lysed sample (100% release) which was prepared by adding distilled water instead of Ringer solution to another portion of packed loaded cells. To determine the possible effect of NaN_3 on the protein and hemoglobin release, a series of release test were carried out with Ringer solution with and without NaN_3, and then, the percent of hemoglobin released was determined in reference to a completely lysed sample.

2.8.2. Hematological indices

The hematological indices of three types of erythrocytes, *i.e.*, IFN-α 2b-loaded, unloaded (normal cells) and sham-encapsulated, obtained from the same subject were determined using a Coulter counter-based instrument (Hematology, model MS9, Sweden). The parameters determined consisted of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin content (MCHC).

2.8.3. Laser-assisted particle size analysis

To investigate the effect of loading process on the particle size distribution of the erythrocyte population, a laser-based particle size analyzer (Shimadzu, model SALD-2101, Japan) was used. For this purpose, IFN-α 2b-loaded, unloaded and sham-encapsulated erythrocytes were analyzed while suspended in saline in a dilution according to instrument operation conditions.

2.8.4. Scanning electron microscopy (SEM)

To investigate the possible morphological changes of erythrocytes upon loading process, samples of three types of erythrocytes were prepared as following stepwise procedure:

1. Fixation in 4% glutaraldehyde and 0.2 M sodium cacodylate.
2. Washing with 0.15 M sodium cacodylate for 10 min, two times.
3. Post fixation in 1% osmium tetroxide and 0.2 M sodium cacodylate, for 2 h.
4. Washing with 0.15 M sodium cacodylate for 10 min, two times.
5. Dehydration with a successive gradient of 35%, 50%, 75%, 95%, and 100% ethanol, each for 10 min.
6. Drying with pure hexamethyldisilazane for 20 min, two times.
7. Transforming the samples to staps.

Table 1
Concentration of IFN-α 2b determined in supernatants and cell lysates upon incubation of intact erythrocytes with isotonic solution of IFN-α 2b[a] ($n = 3$)

Initial added concentration (×1000 IU/ml)	Measured IFN-α 2b concentration (×1000 IU/ml)					
	15 min		30 min		60 min	
	Lysate	Supernatant	Lysate	Supernatant	Lysate	Supernatant
200	4.577 (0.742)[b]	33.774 (1.961)	3.391 (0.658)	34.687 (2.910)	3.856 (0.733)	33.335 (4.044)

[a] Incubation temperature: 25 °C.
[b] Mean (S.D.).

Finally, the prepared samples were analyzed using an electron microscope (SEM 360, Cambridge, UK) after coating with gold particles by a Sputter Coater (Fisons, 7640, UK) in 18 mA for 40 s.

2.8.5. Osmotic fragility

To evaluate the resistance of erythrocytes membranes against the osmotic pressure changes of their surrounding media, 0.1 ml aliquots of the packed samples of each type of erythrocytes, *i.e.*, IFN-α 2b-loaded, unloaded and sham-encapsulated were suspended in 1.5 ml of NaCl aqueous solution having the osmolarities of 0–300 mOsm/l After gentle vertically shaking at 37 °C for 15 min the suspensions were centrifuged at 1000 × *g* for 5 min, and the absorbance of the supernatants was determined spectrophotometrically at 540 nm. The released hemoglobin was expressed as percentage absorbance of each sample in reference to a completely lysed sample prepared by diluting 0.1 ml of packed cells of each type with 1.5 ml of distilled water instead of NaCl solutions. For comparative purposes, an osmotic fragility index (OFI) was defined in each case as the NaCl concentration producing 50% hemoglobin release (Hamidi et al., 2001).

2.8.6. Turbulence fragility

To exploit the mechanical strength of the erthrocytes membranes, 0.5 ml samples of packed erythrocytes of each three types were suspended in 10 ml of PBS and were shaken vigorously using a multiple test tube orbital shaker (IKA, model VIBRAX VXR basic, Germany) at 2000 rpm for 4 h. To determine the timecourse of hemoglobin release, 0.5 ml portions of each suspension were withdrawn in 0, 0.5, 1, 2, and 4 h, and after centrifuging at 1000 × *g* for 5 min, the absorbance of the supernatant was determined spectrophotometrically at 540 nm. The percent release of hemoglobin was determined in reference to a completely lysed cell suspension with the same cell fraction (*i.e.*, 0.5 ml of packed cells added to 10 ml of distilled water). To compare the turbulence fragilities of the different types of erythrocytes, a turbulence fragility index (TFI) was defined as the shaking time producing 20% release of hemoglobin from erythrocytes (Hamidi et al., 2001).

2.9. Data analysis and statistics

The results are presented as mean ± S.D. Statistical comparisons were performed using Student *t*-test or analysis of variance (ANOVA), whenever applicable, using SPSS 12 for windows. The level of significance was taken as 0.05.

3. Results and discussion

3.1. Encapsulation of IFN-α 2b in human erythrocytes

3.1.1. Interferon-α 2b uptake by erythrocytes

The results of incubation of intact human erythrocytes with IFN-α 2b isotonic solutions are shown in Table 1. As indicated in Table 1, no significant amount (maximally about 2%) of IFN-α 2b was taken up by intact erythrocytes. Therefore, it can be concluded that the erythrocyte membrane has no significant active role in the entrapment of IFN-α 2b in human erythrocytes. On the other hand, according to the results in Table 1, a considerable amount (about 83%) of IFN-α 2b was disappeared during the incubation time, which is assumed to be degraded by the intact erythrocytes mainly within first 15 min of incubation. Therefore, caution should be taken when calculating the protein loading or interpreting the results of loading experiments with respect to this degraded amount, whenever applicable. The considerable degree of protein degradation by erythrocytes is in accordance to the well-documented presence of a variety of proteases within the erythrocytes.

3.1.2. Loading parameters

The average loading parameters of IFN-α 2b in human erythrocytes are shown in Table 2. The loaded amount of IFN-α 2b in carrier erythrocytes (Table 2) is relatively comparable to those values reported in the literature for a variety of proteins and peptides (Updike et al., 1976; Pitt et al., 1983; Updike and Wakamiya, 1983; Lizano et al., 2001). This amount, being practically feasible, can ensure sufficient dosing of protein upon re-injection of fairly low volumes of packed cells to the host body. Recently, we have reported the entrapment efficiency of about 30% for bovine serum albumin (BSA) with a molecular weight of about 70 kDa (Hamidi et al., 2007). Though IFN-α 2b is a 20 kDa protein and it was assumed that entrapment efficiency would be better for this smaller molecule, surprisingly, this parameter was lower for IFN compared with BSA. It may

Table 2
Loading parameters of IFN-α 2b in human intact erythrocytes ($n = 3$)

Parameter	Mean	S.D.
Loaded amount (×1000 IU/0.1 ml)	2.906	0.588
Entrapment efficiency (%)	14.53	2.94
Cell recovery (%)	83.61	0.49

Initial IFN concentration of 200,000 IU/ml was used for encapsulation procedure.

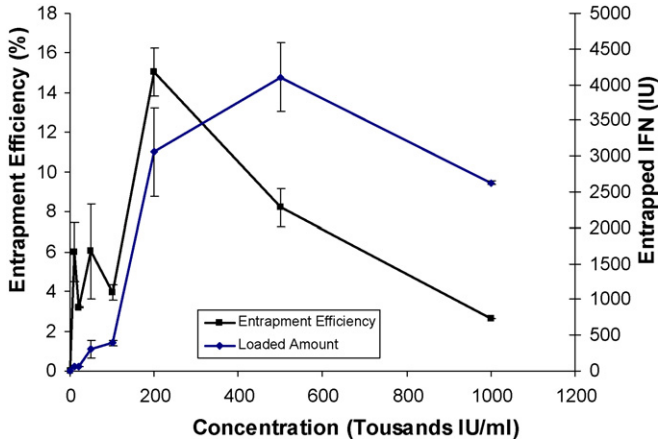

Fig. 1. Effect of IFN-α 2b concentration on loaded amount (IU/0.1 ml) and efficiency of entrapment of IFN-α 2b ($n = 3$).

be concluded that the main reason for this difference could be the higher extent of IFN-α 2b degradation during entrapment procedure. Also, the possibility of the presence of some active efflux pumps for IFN in erythrocyte membrane can not be ruled out. Similarly, low entrapment efficiency (5%) has been reported for insulin as a peptide (Bird et al., 1983). In fact, the results for insulin confirm the impact of peptide/protein degradation (during entrapment procedure) on loading parameters.

Considering the measured loaded amount (about 3000 IU/0.1 ml or 30,000 IU/ml), from one hand, and the predicted fast and extensive RES (as a golden target for IFN action) targeting of these carriers on the other hand, it seems that the loaded amount achieved in this study is appropriate to go further into the next steps, especially *in vivo* studies by means of adjusting the injected volume of IFN-loaded erythrocytes.

A cell recovery of about 83%, being practically appreciable, is better than the result of other studies published by others (*i.e.*, about 70%) (Berman, 1987; Zanella et al., 1987; Al-Achi and Boroujerdi, 1990; Talwar and Jain, 1992a,b; Hamidi et al., 2001).

3.1.3. Effect of concentration of IFN-α 2b solution

The effect of IFN-α 2b concentration on the loaded amount and efficiency of entrapment is shown in Fig. 1. According to Fig. 1, it is clear that the loaded amount of IFN is related directly to the concentration of IFN-α 2b solution used, throughout the concentration range of 10,000–500,000 IU/ml and, then, approaches to a slightly declining phase beyond this range. Although the maximum loaded amount was reached using 500,000 IU/ml drug solution, the efficiency of entrapment was maximal with 200,000 IU/ml drug concentration. It seems that there are some limiting factors against the drug entry into erythrocytes beyond a specific IFN concentration. These factors can be limited binding to some cell surface and/or intracellular sites or presence of some saturable influx transporters. While the use of 200,000 IU/ml drug solution resulted in some lower loaded amount than the 500,000 IU/ml solution, this concentration was selected to use during the process, mainly because of the highest entrapment efficiency with suitable amount of loaded IFN-α 2b in unit volume of packed carrier cells, a parameter that is critical for dose adjustment during *in vivo* studies on this delivery system.

3.1.4. Mechanism of entrapment and method recovery

Data in Table 3 show the measured entrapped and unentrapped amounts of IFN-α 2b in erythrocytes, and Table 4 presents the method recovery in terms of measured-to-expected drug amounts in each compartment.

As shown in Table 3, in an experimental run, a total amount of about 88,000 IU from the total IFN-α 2b added during the entrapment procedure (*i.e.*, 200,000 IU) was discarded during three washing steps. At the same time, the total amount of IFN-α 2b remaining in the erythrocytes was about 21,500 IU per total packed cells recovered after the loading process. The total volume of the reaction mixture was 2.5 ml at the point of resealing. This volume consists of 1.2 ml for swollen cells, 0.2 ml for hemolysate, 1 ml for IFN solution added in four steps, and 0.1 ml for hypertonic resealing solution. From this volume, 0.78 ml (about 32%) belongs to the final carrier cells. Accordingly, it may be expected that, if the distribution of IFN between the intracellular and extracellular fractions would be governed only by a simple concentration gradient-based diffusion, from the total amount of 110,000 IU of added protein remained intact during the entire process, about 35,600 IU would be entrapped within the erythrocytes, and the rest (*i.e.*, 75,000 IU) would be discarded as unentrapped protein. In fact, as shown in Table 4, one can say that the distribution of protein at the lysis point, to the extent of 60% depends on the volume fraction of cells in the suspension, and, thus, it may be concluded that there has been a limiting factor for complete protein entry to erythrocyte at lysis point (*e.g.*, large size, high polarity, and limited diffusion time). On the other hand, as it was shown by the results of the incubation test of intact erythrocytes with IFN-α 2b that the erythrocyte membrane has no significant active role in the uptake of IFN-α 2b, the protein only passes *via* the pores made in the membrane, on hemolysis, inward and outward the erythrocyte compartment. Baker (1967), also, suggested that a transient permeable state of the erythrocyte membrane occurs after the onset of hemolysis. Then, on resealing, the pores are closed, and the drug in

Table 3
Entrapped and unentrapped amounts of IFN-α 2b at the end of the entrapment of protein by hypotonic preswelling method ($n = 3$)

Fraction	Volume (ml)	IFN-α 2b concentration (×1000 IU/ml)	IFN-α 2b total amount (×1000 IU)
Total washing solution	28	3.143 (0.324)[a]	88.013 (9.084)
Final packed cells	0.7816 (1.177)	27.627 (0.6718)	21.593 (5.250)
Total amount recovered			109.607 (7.707)

[a] Mean (S.D.).

Table 4
Recovery of IFN-α 2b as entrapped, unentrapped, and total IFN-α 2b after the entrapment of protein by hypotonic preswelling method ($n = 3$)

Fraction	Expected amount (×1000 IU/ml)	Measured amount (×1000 IU/ml)	Recovery[a]
Entrapped fraction	65.600	21.593 (5.250)[b]	32.92% (8.01)
Unentrapped fraction	134.400	88.013 (9.084)	65.49% (6.76)
Total	200.000	109.607 (7.707)	54.80% (3.86)

[a] The percentage ratio of measured amount to expected amount of IFN-α 2b in each fraction.
[b] Mean (S.D.).

'entrapped' in the erythrocytes. The mechanism proposed here is in agreement with results published by others (Baker, 1967; Ihler et al., 1973; Kinosita and Tsong, 1977).

Comparing the degraded amount (about 45%) in this experiment with degraded amount (about 83%) found in incubation test and considering the fact that erythrocytes are completely intact during incubation, it could be concluded that IFN-α 2b was degraded more extensively by intact erythrocytes compared to those erythrocytes undergone entrapment procedure. It may be some degrading systems susceptible to entrapment condition, active in IFN loss during the incubation period.

The recovery of the entrapment process was shown in Table 4 with respect to entrapped, unentrapped, and total protein. These data, while providing reasonable basis for investigation of the mechanism of entrapment, as discussed earlier, indicate a degree of recovery of about 55% for IFN throughout the whole process.

3.2. Process validation tests

The intra-subject variations (CV%) of the loading procedure were 6.55% and 6.57% for loaded amount and efficiency of entrapment, respectively. The corresponding values for inter-subject variations were 7.55% and 7.56% for men and 13.63% and 13.64% for women. Generally, the entrapment efficiency was lower in females but this difference is not significant ($P > 0.05$).

3.3. In vitro characterization of IFN-α 2b-loaded erythrocytes

3.3.1. IFN and hemoglobin release

The release profiles of IFN-α 2b and hemoglobin from carrier erythrocytes while being shaken at 37 °C are shown in Fig. 2.

From the drug and hemoglobin release profiles shown in Fig. 2, it can be concluded that:

(i) IFN release is a somehow rapid process compared to cell lysis kinetics, represented by hemoglobin release. The drug release approaches to about 100% at 4 h as opposed to the hemoglobin release of about 10% at the same time. This behavior is surprisingly different from all experimental results reported on similar protein/peptide drugs, including BSA (Hamidi et al., 2007), asparaginase (Updike et al., 1976; Updike and Wakamiya, 1983), and enalaprilat (Hamidi et al., 2001) as well as other polar drugs such as heparin (Erchler et al., 1987) and gentamicin (Erchler et al., 1986). This observation can be best explained by the presence of some efflux transporters in erythrocyte membrane mediating IFN active efflux out of the cells.

(ii) Considering the relatively rapid drug release (leak out) from carrier cells, it seems that there may be a need for some release controlling factors (*e.g.*, membrane stabilizers) such as glutharaldehyde for achievement of practically feasible RES targeting of IFN using this carrier system.

(iii) The declining trend of released IFN from 24 to 48 h of incubation can be explained, mainly, by the presence of some 'destabilizing factors' for IFN, released from erythrocytes within this time period.

(iv) The hemoglobin release trend, while obeying an almost zero-order kinetics from 0 to 24 h, approaches a plateau in 24 h at 80% lysis, which can be explained by the presence of some 20% of the cells remained intact during the destructive loading procedure used.

Also, addition of NaN_3, as microbial preservative, has not significant effect on percent of released hemoglobin ($P > 0.05$). Therefore, NaN_3 exerts no effect on release profile of hemoglobin and, can be used as a suitable preservative in this case.

3.3.2. Hematological indices

The hematological indices of the tested human erythrocytes are shown in Table 5.

From the data in Table 5, no significant changes are evident with the entrapment process alone or together with IFN-α 2b loading, on the mean volume of erythrocytes ($P > 0.05$). However, both MCH and MCHC were decreased following exposure

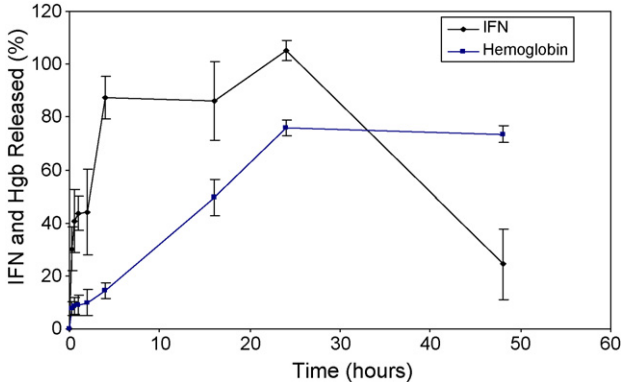

Fig. 2. The release profiles of IFN-α 2b and hemoglobin from carrier erythrocytes ($n = 3$).

Table 5
Hematological indices of three types of erythrocytes ($n = 3$)

Hematological index	Unloaded erythrocytes	Sham-encapsulated erythrocytes	IFN-loaded erythrocytes
MCV[a] (fl)[b]	94.7 (0)[c]	94.6 (0.4)	95.9 (1.2)
MCH[d] (pg)[e]	32.7 (1.6)	18.5 (0.6)	24.6 (2.5)
MCHC[f] (g/dl)[g]	35.3 (1.7)	19.65 (0.8)	23.7 (1.9)

[a] MCV: mean corpuscular volume.
[b] fl: femtoliter.
[c] Mean (S.D.).
[d] MCH: mean corpuscular hemoglobin.
[e] pg: picogram.
[f] MCHC: mean corpuscular hemoglobin content.
[g] g/dl: gram per deciliter.

of the erythrocytes to entrapment procedure with or without IFN-α 2b loading, with the extent of decrease being higher in sham-encapsulated group ($P < 0.05$). This observation can be explained by some protective effect, as a physical or functional barrier, of IFN-α 2b on hemoglobin loss from carrier erythrocytes.

In other studies (Baker, 1967; Deloach and Doleskey, 1987; Garin et al., 1996; Hamidi et al., 2001), all these parameters were decreased in carrier erythrocytes compared to the normal unloaded cells. Our results showed that the loading procedure did not affect mean cell volume significantly, since the MCV values remain in normal range in all the cases. This finding, being supported by our laser-assisted particle size analysis data (see below), is in agreement with the results of Hamidi et al. (2001). The decreased hemoglobin content of the carrier cells is quite reasonable, considering the destructive nature of the loading method used, which leads to a remarkable degree of hemoglobin loss in final wash solutions.

All these results for IFN-α 2b, are in close agreement with those obtained for BSA using the same method by our group (Hamidi et al., unpublished data).

3.3.3. Laser-assisted particle size analysis

The statistical parameters derived from volume- and number-based diameter distribution curves of three types of erythrocytes show that the particles are unidisperse (unimodal curves) and normally distributed in unloaded, sham-encapsulated and IFN-loaded cells. The mean (5.634 ± 0.075 μm for number-based and 6.185 ± 0.078 μm for volume-based data), median (5.604 ± 0.193 μm for number-based and 6.209 ± 0.219 μm for volume-based data), and modal (5.623 ± 0.000 μm for number-based and 6.078 ± 0.000 μm for volume-based data) diameters of IFN-loaded erythrocytes remain without any significant changes after the loading process ($P > 0.05$). Furthermore, the size dispersity of the erythrocytes population is statistically the same in three types of erythrocytes ($P > 0.05$). This means that the loading procedure has no significant effect on particle size and size distribution of the erythrocytes. This effect, being consistent with our measured MCV values, is in contrast with the previous report that claimed enlargement of erythrocytes during the loading procedure with higher dispersity of cell diameters (Hamidi et al., 2001).

3.3.4. Scanning electron microscopy

In Fig. 3, the scanning electron micrograghs of erythrocytes of three types are shown in different magnifications. As illustrated in Fig. 3, the loading process, with and without the IFN-α 2b entrapment, resulted in the formation of erythrocytes with very dispersed sizes and shapes. Different stages of biconcave, cup-form, stomatocyte (a particular from of spherocytes with an invagination in one point), spherocyte (spherical erythrocytes), echinocyte and irregular-shaped cells are evident in Fig. 3B and C. These findings show that erythrocytes undergo considerable morphological changes during the entrapment process, which was predictable based on the destructive nature of the loading process. Furthermore, from these micrographs, we can conclude that inclusion of IFN-α 2b itself has no observable additional effect on the morphology of the carrier cells and that the observed changes are due to the entrapment process by itself.

Other studies have reported the same findings. SEM studies by other groups has revealed that the majority of these cells maintain their normal biconcave discoid shapes after exposure to the loading procedure (Deloach and Doleskey, 1987) with some "spherocytes" (Garin et al., 1996) and a few "stomatocytes" and, in some cases, a few microcytic erythrocytes (erythrocytes with smaller sizes than the normal cells) (Kruse et al., 1987). Hamidi et al. (2001) reported the presence of cup-formed cells with different sizes as well as stomatocytes in the samples of carrier erythrocytes analyzed by SEM technique. It is obvious that the extent of irreversible shape changes occurred in carrier erythrocytes compared to normal cells, is a function of the loading method used which, in turn, exert different degrees of changes in erythrocytes shape and surface properties. The highly changed erythrocyte shape and topology evidenced in this study, being one of the main determinants in erythrocytes disappearance kinetics in circulation, can be potentially beneficial in terms of successful cell targeting to RES, which, in turn, leads to the improved IFN effects on RES-mediated immune responses. One of the most profound effects of loading procedure on cellular shape, as seen in SEM photographs, is the generation of cells with considerably smaller sizes compared to the normal unloaded cells. These microcytic cells result from the cell structural regeneration during the resealing step of the loading process. These so called 'erythrosomes' are indicative of a destructive method for loading, which is suitable only for RES targeting purposes. The sizes evident in this test, which is

Fig. 3. SEM photographs of (A) unloaded (magnification of ×5000), (B) sham-encapsulated (magnification of ×2000), and (C) IFN-loaded (magnification of ×2000) erythrocytes.

opposed to the findings of MCV (see Sections 2.8.2 and 3.3.2) and laser-assisted particle size analysis section of this study, can be explained by the formation of somehow permanent cell aggregates between the erythrocytes which, in turn, causes some false 'apparent shapes' despite the smaller real particle shapes.

3.3.5. Osmotic fragility

The osmotic fragility curves of three types of erythrocytes are shown in Fig. 4. The osmotic fragility indices of unloaded, sham-encapsulated, and IFN-α 2b-loaded erythrocytes, being 147, 175, and 195, respectively, indicate that the loading process with and without protein entrapment results in more fragile cells against the osmotic pressure changes. The lower resistance of the carrier cells to osmotic changes is a measure of the loss of integrity of the erythrocyte membrane and its natural behavior as a result of loading procedure. In addition, incorporation of IFN-α 2b in cells increases the osmotic fragility of the cells, a result with no explanation within the scope of this study. It has been reported similarly previously by Hamidi et al. (2001), the trend of osmotic fragility curve, changes from sigmoidal in the case of unloaded cells to linear in the case of both IFN-α 2b-loaded and sham-encapsulated erythrocytes. This is indicative of more heterogeneous cell population in terms of osmotic fragility in the cases of IFN-α 2b-loaded and sham-encapsulated cells. Such heterogeneity is also evident in our SEM micrographs in terms of the cell shape and surface features. In most of the studies testing this parameter, osmotic fragility of the carrier erythrocytes has been higher than the normal unloaded cells along with a change in the trend of fragility curves from sigmoidal to some linear curves (Kinosita and Tsong, 1977; Talwar and Jain, 1992a,b; Garin et al., 1996; Jain et al., 1997; Hamidi et al., 2001).

Fig. 4. Osmotic fragility curves of unloaded, sham-encapsulated, and IFN-α 2b-loaded erythrocytes ($n = 3$).

3.3.6. Turbulence fragility

The results of turbulence fragility test on three types of erythrocytes are shown in Fig. 5. From these curves, the values of turbulence fragility indices (TFI) for unloaded, sham entrapped and IFN-α 2b-loaded erythrocytes are 3.68, 2.26, and 1.18, respectively. According to these results, the resistance of the erythrocytes against the vigorous turbulent flow shows a decreasing trend from unloaded cells to IFN-α 2b-loaded erythrocytes. This

Fig. 5. Turbulence fragility curves of unloaded, sham-encapsulated, and IFN-α 2b-loaded erythrocytes ($n = 3$).

test is indicative of production of more fragile erythrocytes due to the changes in integrity of the erythrocyte membrane upon loading process alone and together with IFN-α 2b-entrapment. As mentioned before, the results of other studies have also shown that turbulence fragility of the carrier erythrocytes increases in comparison to the normal unloaded cells (Talwar and Jain, 1992a,b; Jain et al., 1997; Hamidi et al., 2001). The resistance of the erythrocytes against the vigorous turbulent flow shows a decreasing trend from sham-encapsulated to IFN-α 2b-loaded erythrocytes with no more explanation in this context. This trend also was seen during the osmotic fragility test and this similarity is probably a result of some deviation of the carrier erythrocytes from their normal state, an effect that became more pronounced in the presence of IFN-α 2b during the loading process. The main impact of turbulence fragility in erythrocyte fate in the body is expected during the cell passage through the extreme capillaries as well as the spleen trabecules.

4. Conclusion

The human erythrocytes were loaded successfully with IFN-α 2b with the practically acceptable loading parameters. The loaded cells were evaluated with respect to their *in vitro* drug delivery characteristics, including drug and hemoglobin release, hematological indices, particle size distribution, shape and surface properties, osmotic and turbulence fragilities. The results of these tests, collectively, were indicative of some irreversible changes in cell morphology as well as physiology, which, in turn, may favor the cell targeting to RES organs. The relative impact of each of these changes remains to be exploited in ongoing animal studies in our laboratory.

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