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MACROMOLECULAR COMPOUNDS AND POLYMERIC MATERIALS

Impacts of Chemical Variables on the Encapsulated Corticoids in Poly-ε-caprolactone Nanoparticles and Statistical Biological Analysis[1]

R. Amiri Dehkharghani[a,*], M. Zandi Doust[a], M. Tavassoti Kheiri[b], and H. Hossein Shahi[a]

[a]*Department of Chemistry, Central Tehran Branch, Islamic Azad University, Tehran, Iran*
**e-mail: rahebeha@gmail.com*
[b]*Department of Influenza Unit, Pasteur Institute, Tehran, Iran*

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Abstract—The goal of this research is to evaluate the impact of different parameters on corticosteroids entrapment in biocompatible poly-ε-caprolactone nanoparticles. These findings provide better insight on the designing carriers for drugs. Nanocapsules were synthesized by interfacial deposition and their morphology was determined by SEM. Drug entrapment efficiency and particle size distribution were assayed by HPLC and DLS, respectively. The samples were assessed for cytotoxicity using MTT reduction assay. The anti-inflammatory effect of the formulated drug was determined by induction of inflammation in treated as well as native laboratory animals. Statistical analysis of the data was performed using SPSS 18.0.

Keywords: corticoids; poly-ε-caprolactone; nanoparticle; encapsulation; inflammation

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Corticoids are used as therapeutic agents in pharmacological treatment of some diseases such as eczema, fibrosis, and ocular diseases associated with inflammation [1]. Unfortunately, the use of corticoids is often hampered by the induction of side effects such as osteoporosis, hyperglycemia, glaucoma, high blood pressure, depression, etc. To overcome these problems, the incorporation of drugs into nanoparticles represents more effective therapeutic opportunity [2]. Nanoparticles based on biodegradable polymers such as aliphatic polyester are being widely investigated as delivery systems for different drugs [3]. They can successfully transfer the drug to target site and increase the therapeutic benefit or minimize the side effects [4–8]. The advantage of aliphatic polyesters such as poly-ε-caprolactone is their ability to hydrolyze to toxicologically safe compounds which are further eliminated by the normal metabolic pathways [9]. On the other hand, various methods have been also reported for the successful preparation of the nanoparticles [10]. Interfacial deposition technique is based on the emulsification of an organic phase with the dissolved polymer into the aqueous phase and emulsifier. The polymer containing drug (encapsulated drug) is deposited on the interface between water and the organic solvent [11]. In this method, the model of drug incorporation into PCL is the drug-enriched core [12]. Several factors of manufacturing conditions may affect drug loading content and encapsulation efficiency such as concentration, and type of drug, injection rate, temperature, and pH [13, 14]. Therefore the parameters were assessed to find the most effective one. Finally, in vitro and in vivo release of the optimized formulation was evaluated under biological conditions. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay was used to detect toxicity effect of the PCL nanoparticles. Groups of rats were injected with the drug alone or in the nanocapsule form followed by intradermal injection of the Complete Freund Adjuvant in the animal footpad

[1] The text was submitted by the authors in English.

to induce inflammation. The edema was measured and compared among these groups.

EXPERIMENTAL

The polymer used for the encapsulation was poly-ε-caprolatone (Aldrich chemical, USA). Its average molecular weight given by Aldrich was close to 80 000 Da. All the materials were purchased from Sigma, USA. All the solvents were HPLC grade. The morphology of the prepared nanocapsules was examined with a Scanning Electron Microscope (JEOL, JMS-840). Particle size distribution of the nanocapsules were determined by dynamic light scattering (DLS) using a zetasizer Nano instrument (Malvern Instruments, Nano Zs, ZEN 3600, UK) operating with a laser 532. The spectra in the UV-Vis range of 200–800 nm were taken using spectrophotometer V570 Jasco. The high performance liquid chromatography (HPLC) was used for the determination of drug loading content. The chromatographic system consisted of a Gemini RP-18 column (150 mm × 4.60 mm, 5 μm, phenomenex, Torrance, USA) and a Shimadzu instrument (LC-10AVP Pump, UV-Vis SPD-10AVP Module, Class Vp-Software, Shimadzu, Tokyo, Japan). J774. A1 were seeded at 20 000 cells per well in complete medium containing 10% FBS. The absorbance Optical Density (OD) was measured at 540–690 nm on a Microplate reader Labsystems Multiscan, Stat Fax-200.

For the synthesis of poly-ε-caprolactone nanocapsules containing drug, an organic solution composed of hydrocortisone (0.0125 g), the oily phase, SFO (0.8 mL), sorbitan monooleate (0.194 g), the polymer (PCL) (0.25 g), and acetone (67.0 mL) was added to an aqueous solution (134.0 mL) containing polysorbate 80 (0.194 g) under moderate magnetic stirring (10 min). The pH of the final suspension was 7. Then, the acetone was eliminated by evaporation under reduced pressure (40°C). The suspension was centrifuged (9500 rpm) for 10 min and then the samples were put in refrigerator for 2 h. The solid phase was separated by filtering and then was washed.

Vero cells (CCL-81) which are lineages of immortalized normal cells, were used in cell culture study. They represent triangular shape in normal condition while "round off" in poisonous condition. Thus, they are used to test the toxicity effect of chemicals going to be used as a drug.

Vero cells were cultured as a monolayer in sterile culture flasks containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% pen/strep antibiotic. A cell suspension containing 3×10^4 cells/mL was seeded in 96-well microliter plates and incubated at 37°C in a CO_2 humidified incubator. All freshly prepared solutions were serially two-fold diluted (1, 0.5, 0.25, 0.125, and 0.0625 mg mL^{-1}) and added to the assigned wells.

The female rats which were used in this study (170–200 g weight) were purchased from Pasteur Institute of Iran and housed in a temperature-controlled environment (20–22°C). Rats had free access to water, standard laboratory food and other conditions. The amount of 600 mg of pure powder of drug dissolved in 5 mL PBS was used to prepare the solutions under optimal and sterile conditions. The amount of 5 mL of all the solutions (1.02 mg kg^{-1}) was injected to rats. Subcutaneous injection of 0.3 mL FCA into the right paw of each rat led to inflammation, pain, licking and biting of the paw.

Statistical analysis of the data was performed using SPSS 18.0. Data were presented as mean ± SD. A one-way analysis of variance (ANOVA) post-hoc LSD test was used to analyze the data for cytotoxicity and biological activity. For all the tests, $P \leq 0.05$ was considered as significant.

RESULTS AND DISCUSSION

The effect of chemical variables. The drug concentration in nanocapsules was determined on a UV-Vis spectrometer. The ultraviolet-visible (UV-Vis) spectra of hydrocortisone and prednisolone, in acetonitrile (15.4 mg mL^{-1}) showed the maximum absorption at 232 and 240 nm wavelength ($\log \varepsilon = 3.35$ and 1.42) for them, respectively. The drug concentration was varied from 5.0 to 24.5 mg mL^{-1} while other processing parameters were kept constant. According to the results, the concentration of drug with the maximum absorption (similar to pure drug) was obtained at 12.5 mg mL^{-1}. However, the differences in concentrations and the type of drug (hydrocortisone or prednisolone) have not shown any significant effect on drug loading. Another study was performed to evaluate the effect of injection rates of two oily and aqueous phases on the maximum absorption of the nanocapsules. This factor could change the size of nanocapsules and subsequently, the amount of encapsulated drug. The desired injection rates were achieved using different nitrogen pressures from 0.1 to 3 bar. The pressure of N_2 gas with the maximum absorption was obtained at 2.5 bar for

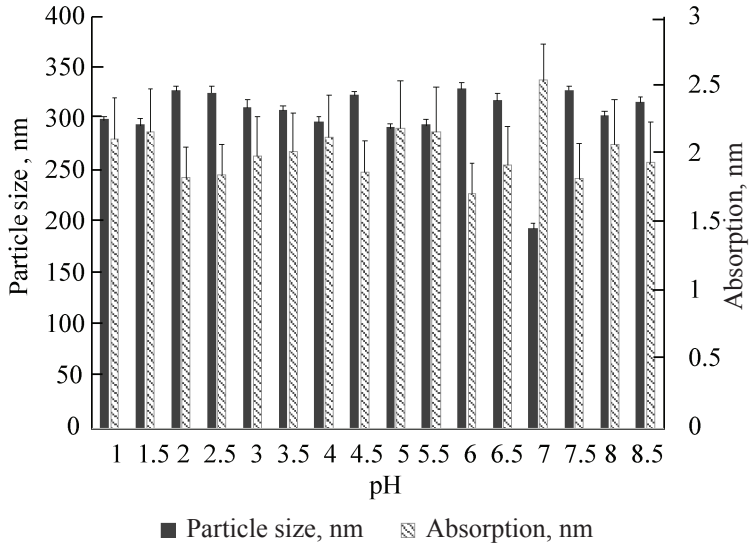

Fig. 1. The effect of pH on the maximum absorption of nanocapsules and their particle size.

both hydrocortisone and prednisolone. However, it did not make much difference, and consequently, we found the injection rate in this method (interfacial deposition) was not effective in trapping of the drug. Viscosity is one of the properties of polymers, which can rapidly change with temperature, so this parameter could lead to precipitation of the polymer instead of the capsules constitution. The amount of drug in nanocapsules could be a useful measurement of the nanocapsules formation. The temperature of aqueous phase was raised from 10 to 40°C (during the injection of two phases to each other). The maximum absorption of the resulting nanocapsules was observed at 30°C without any significant changes or specific trend that associate with the type of drug. For investigating the rate of pH effect on the maximum absorption of the encapsulated drug, some different pH (from 1 to 8.5) was also tested. Figure 1 shows that pH = 7 is the optimum pH with the maximum absorption of the nanocapsules for both drugs. In addition, the particle size of nanocapsules at this pH is the lowest. Since, the lower solubility of hydrocortisone or prednisolone in water especially at neutral pH can lead to its absorption into the polymeric matrix system of nanoparticles, so there is an increase in drug loading at pH = 7. Furthermore, in the case of the anionic network (e.g., polycaprolactone with –COOH group), when the pH value of the surrounding medium is greater than the pK_a value of the acidic groups on the polymer chains, ionization of them takes place leading to production of fixed negative charges on the polymer chains and then the electrostatic repulsion between the chains leading to creating the more space to load the drug. On the other hand, if steric effect is the main stabilizing mechanism, the particle size will not be very sensitive to pH. However, we here observed that the particle size of nanocapsules is sensitive to pH changes (Fig. 1). Thus, electrostatic stabilization is the main reason to reduce the particle size because the solution pH can markedly affect the protonation degree of anionic poly-ε-caprolatone and consequently, a proper pH can prevent the agglomeration of particles. Also, the results are similar for both hydrocortisone and prednisolone (due to structural similarity). Therefore, it is clear that the degree of pH impact is more significant on encapsulation of corticoids in poly-ε-caprolatone.

Characterization of nanocapsules. The morphology of the nanocapsules obtained from the optimal conditions was determined by SEM. Scanning electron microscopy was also used to establish a second opinion on nanoparticle size distribution. The spherical shape and a smooth surface of nanocapsules can be seen in Fig. 2. The SEM image showed no aggregate polymer formation and the size of nanocapsules were below 200 nm.

Dynamic light scattering (DLS) is widely used to determine the size of Brownian nanoparticles in colloidal suspensions. It has been found that particle size will affect the drug release, the physical stability and the cellular uptake. Smaller particles offer larger surface area and it leads to fast drug release [15]. The nanocapsules containing any of the drug were of the mean diameter of

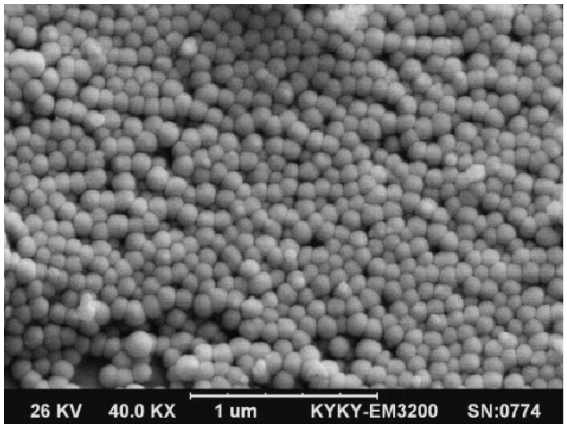

Fig. 2. SEM image of the final nanocapsules containing drug.

Table 1. Prepared solutions for MTT assay and biological activity

Group	Components
S_0	Hydrocortisone dissolved in PBS
S_1	Hydrocortisone in freshly prepared nanocapsules suspension
S_2	Freshly prepared nanocapsules suspension without hydrocortisone
S_3	Hydrocortisone dissolved in acetone and distilled water
PBS	Phosphate salt solution

194 ± 58 nm, negative zeta potentials (–30.3 ± 0.25 mV) as well as polydispersity indices (0.14 < 0.2) indicating an adequate homogeneity of this system. Extremely negative zeta potential value causes larger repulsive forces and thus prevents aggregation of the particles, which confirms the electrostatic stability of this system. A rapid, specific and reliable HPLC method has been developed and validated for the assay of corticoids in topical nanocapsule suspensions. The analytic methodology proposed is simple, precise, accurate, and linear in the concentration range of 2.0–20.0 μg mL^{-1}. The proportion of the mobile phase (acetonitrile–water) was evaluated 80 : 20 (v/v). This proportion showed retention times for hydrocortisone and prednisolone of 1.67 and 1.71 min ,respectively.

Drug entrapment efficiency (EE% = 90% ± 0.7) was calculated using Eq. (1).

$$EE = \frac{\text{Weight of drug in nanoparticles}}{\text{Weight of drug fed initially}}. \quad (1)$$

Cytotoxicity assay (MTT). In vitro cytotoxicity test is a function of cellular metabolism against toxic chemicals and toxicity can be measured by assessing cellular damage [16–18]. Indirect methods which use colorimetric reagents are currently among the most attractive methods. The parameter used as the basis for colorimetric assays is the metabolic activity of viable cells (the more viable cells, the less toxicity of material). The MTT assay is a colorimetric assay that relies on the enzymatic reduction of a yellow tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), which forms purple formazan crystal in metabolically active cells [19]. It can then be solved and produce a colorimetric signal at 540–690 nm. The concentration of formazan produced depends on the cells number and their activity [20]. So, a variety of concentrations of S_1, S_2, and S_3 solutions (Table 1) were added to the cells.

The optical densities (ODs) were measured using micro-plate reader and the percentage of cell viabilities was calculated using the OD information of the sample as a ratio to negative control. The cell viabilities in 20, 44, and 68 h and different concentrations of the desired material were calculated using ODs according Eq. (2)

$$\text{Cell viability} = (OD_{test} - OD_{blank})/(OD_{control} - OD_{blank}), \quad (2)$$

The results obtained from this study demonstrated that the drug in our formulations shows a little toxic effect at concentrations of 1 and 0.5 mg mL^{-1}. In spite of that, the particles with the lowest zeta potential values (the most negatively charged) are phagocytized in a higher extent by macrophages [21], no measurable toxicity was observed at lower concentrations; and even an increase in viability of the cells was detected in some cases (Fig. 3).

Biological activity. Since, the skin has positive zeta potential, the opposite charge may increase contact time between drug and skin, and this electrostatic interaction could be a technological advantage [22, 23]. So, the purpose of this study was to evaluate the effect of synthetic nanocapsules (with negative charge) containing drug to reduce inflammation in animal models. Therefore, five groups of rats (A–E in Table 2) were injected by FCA in footpad to induce inflammation and edema [24, 25].

Assessment of the edema was performed using a vernier caliper. Increasing volume and thickness of the

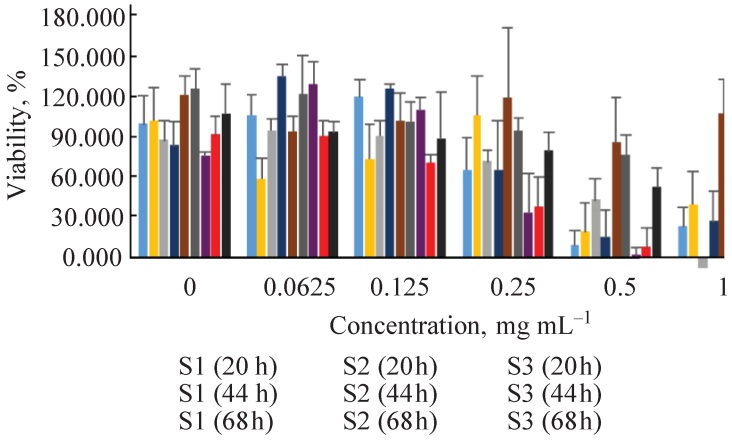

Fig. 3. Mean MTT results of treatments with different concentrations of prepared solutions.

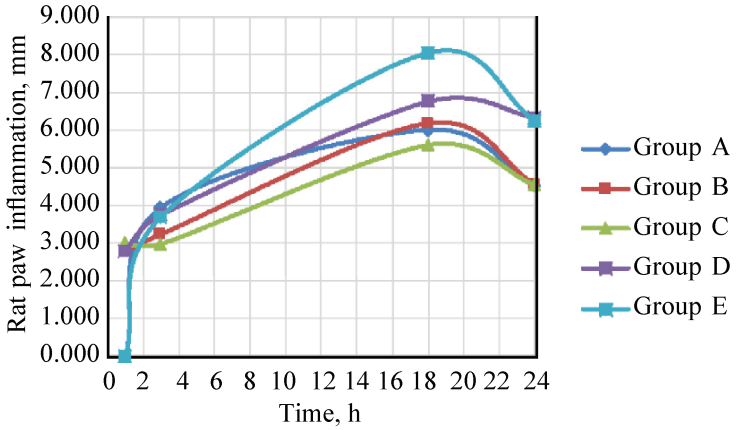

Fig. 4. The graphical comparison of inflammation at different times.

paw in the upper, lower and anterior parts were measured at day zero (immediately after injection of adjuvant) and 1, 3, 18, 24 h after injection of FCA [26–30]. Inflammation of the subcutaneous injection of complete Freund's adjuvant in untreated female rat paw was compared with the rats treated with drug. Measurements of inflammation with caliper showed that the inflammation in all groups, regardless of the type of drug, was decreased after 18 h. The group received PBS (phosphate salt solution) as control for inflammation, were compared with other

Table 2. Determination and comparison of the rat paw inflammation by measuring edema of the injected paw (mm)

	Paw inflammation, mm, (Mean ± SD)			
Group	1 h	3 h	18 h	24 h
A (S_0)	ND[a]	3.94 ± 1.21	6.00 ± 0.74[b]	4.50 ± 0.87[b]
B (S_1)	2.79 ± 0.20	3.24 ± 0.16	6.18 ± 0.50[b]	4.56 ± 0.39[b]
C (S_2)	3.00 ± 0.19	2.98 ± 0.14	5.59 ± 0.56[b]	4.54 ± 0.68[b]
D (S_3)	2.80 ± 0.31	3.76 ± 0.27	6.76 ± 0.34[b]	6.24 ± 0.42
E (PBS)	2.70 ± 0.32	3.70 ± 0.46	8.06 ± 0.34	6.36 ± 0.49

[a] ND: Not determined. No significant difference was observed for solutions-treated samples compared to untreated sample (PBS) ($p \geq 0.05$) in 1 and 3 h.

[b] Significantly different compared to untreated sample ($p < 0.05$) in 18 and 24 h.

groups. It was found that the drug both alone and in nanocapsules reduced the inflammation. Thus, it was shown that the anti-inflammatory effect of drug has not been reduced in the formulation used in our study (Fig. 4).

CONCLUSIONS

In the study, we have performed the nanocapsulation of corticosteroids through interfacial deposition method. We examined some factors in the procedure and the optimum pH, injection rate, concentration of drug and temperature were obtained as 7, 2.5 bar, 12.5 mg mL^{-1} and 30°C, respectively. In addition, we compared the impact of these factors on encapsulation and found that the size of nanoparticles composed of anionic polymer and drug loading were obviously pH-dependent. Drug entrapment efficiency (90% ± 0.7) was assayed by HPLC. The results obtained from DLS comply with the results of SEM and the highest particle size distribution in a given volume has been assessed around 200 nm. Therefore, it can be concluded that interfacial deposition at neutral pH is a suitable method for encapsulation of corticosteroids in poly-ε-caprolactone and the other chemical variables have little effect on the outcome of work. In order to investigate the toxicity of the produced nanoparticles, MTT assay was performed and the results showed that the formulation is not only non-toxic at lower concentrations, but also increases the cell viability. It was observed that corticosteroids alone as well as in the synthetic formulation have reduced inflammation after 18 h. In parallel with our experiments, we tested suspension that was synthesized 48 h before the injection to rats that showed similar results to freshly prepared suspension. Therefore, in this step we could justify that anti-inflammatory effect of the drug in the synthetic formulation did not decrease and the drug effect was not disrupted ($p \geq 0.05$).

However, it worth to mention that further investigation is possible by testing different formulations for dose sparing of the drug. Also, developing more studies to evaluate the synergism effects and targeting could be tested.

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