

Research Article

Cytotoxicity and Biological Efficacy of Exendin-4-Encapsulated Solid Lipid Nanoparticles in INS-1 Cells

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Received 11 June 2015; Accepted 24 August 2015

Academic Editor: Aran Son

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Exendin-4 (Ex-4), a peptide of glucagon-like peptide-1 receptor agonist, is a potent insulinotropic agent and alternative drug delivery systems to increase therapeutic utility have been explored. We developed exendin-4-encapsulated solid lipid nanoparticles (Eudragit Ex-4 SLNs) and compared the effects of Eudragit Ex-4 SLNs with those of native Ex-4 on INS-1 cells. We observed no significant toxic effects of nanoparticles at concentrations from 1 nM to 100 nM. Similar to Ex-4, Eudragit Ex-4 SLNs stimulated the production of cyclic AMP at 10 nM. Moreover, unlike treatment with the vehicle, treatment with 10 nM Eudragit Ex-4 SLNs increased insulin mRNA levels and insulin secretion. These insulinotropic effects of Eudragit Ex-4 SLNs were comparable to those of Ex-4. Thus, our *in vitro* results suggest that the biological effects of Eudragit Ex-4 SLNs are similar to those of Ex-4, and further *in vivo* pharmacokinetic studies are required to propose an alternative sustained release drug system.

1. Introduction

Exendin-4 (Ex-4), the first clinically available incretin mimetics, is an analog of glucagon-like peptide-1 (GLP-1) [1]. Ex-4 is resistant to dipeptidyl peptidase-IV and thus is 3000-fold more effective than GLP-1 [2]. Ex-4 is a GLP-1 receptor agonist with several glucoregulatory effects such as glucose-dependent increase in insulin secretion, suppression of glucagon secretion, and reduction of gastric mobility and food intake [3, 4]. Exenatide, a synthetic version of Ex-4, is approved as a promising therapy for the treatment of patients with type 2 diabetes [5, 6]. Exenatide is administered via frequent subcutaneous (SC) injections; therefore, an alternative route of administration such as the oral route is being examined to increase its therapeutic utility [7, 8]. Recent studies have shown that nanosized drug delivery systems increase solubility, decrease side effects, prolong pharmacological effects, and improve bioavailability [9, 10]. Solid lipid nanoparticles (SLNs) are particles made from crystalline solid lipids and have a mean diameter of 50~1000 nm [11]; their advantages include controlled drug release

and drug targeting, protection of the incorporated compound against chemical degradation, lack of carrier biotoxicity, and no problems associated with large-scale production [11–13]. In recent studies, different combinations of solid lipid materials having various physicochemical properties have been examined for encapsulation of peptide drugs [14, 15]. To develop an extended delivery system for Ex-4, we used solid lipid nanoparticles for encapsulation of Ex-4. In this study, we prepared Ex-4-loaded SLNs using Precirol, and we first compared its biological effects with those of native Ex-4 in INS-1 beta cells before further study. We found that biological effects of Ex-4-loaded SLNs are comparable to those of Ex-4.

2. Methods

2.1. Reagents. Precirol was obtained from Gattefosse (Gennevilliers, France) and Eudragit L30 D55 with PlasAcryl HTP 20 was purchased from EVONIK (Lafayette, Indiana, USA). Native Ex-4 was from Tocris Bioscience (Bristol, United Kingdom). Insulin enzyme immunoassay (EIA) kits were purchased from Alpco Diagnostics (Windham, NH,

USA) and competitive cyclic adenosine 3'5'-monophosphate (cAMP) immunoassay kit was from Enzo Life Sciences (Plymouth Meeting, PA, USA). All other biochemical reagents, unless otherwise specified, were obtained from Sigma (St. Louis, MO, USA) or Invitrogen (Carlsbad, CA, USA).

2.2. Cell Culture. INS-1 cells were derived from a rat insulinoma and were maintained in a monolayer culture in Roswell Park Memorial Institute- (RPMI-) 1640 medium containing 11 mmol/L glucose. The culture medium was supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin.

2.3. Preparation of Eudragit-Coated Ex-4 SLNs. Precirol was dissolved in dichloromethane and the mixture with Ex-4 (internal water phase) was dispersed by ultrasonication (Vibracell VCX130; Sonics, USA) at 20 amp, 2 pulses, to form a primary water-in-oil (W/O) emulsion. A double emulsion (W/O/W) was formed after addition of Tween 80 solution followed by sonication at 20 amp, 2 pulses. The organic solvent was evaporated using a rotary evaporator thus producing the SLN dispersion. This SLN dispersion was mixed with 1% of equal volume of Eudragit (Eudragit L30 D55 with PlasAcryl HTP 20) under magnetic stirring at 600 rpm for 10 min. The resulting formulation was Eudragit-coated Ex-4 SLNs (Eudragit Ex-4 SLNs) and Eudragit blank SLNs were used as controls.

2.4. Cytotoxicity Assay of Nanoparticles Using INS-1 Cells. We determined the cytotoxicity of Eudragit Ex-4 SLNs or Eudragit blank SLNs in INS-1 cells by examining the dehydrogenase activity. The cells were plated in 96-well plates and were incubated with various concentrations of Eudragit blank SLNs and Eudragit Ex-4 SLNs (final concentrations: 1~100 nM) at the indicated times. The number of viable cells was examined using the cell counting kit-8 (CCK-8) assay kit (Dojindo Lab., Kumamoto, Japan). Briefly, 10 µL of CCK-8 solution was added to each well and the plate was incubated at 37°C for 2 h. The absorbance of each well was measured at 450 nm using a microplate reader (Molecular Devices Corp., Menlo Park, USA).

2.5. Measurement of Intracellular cAMP Levels. INS-1 cells were plated on 12-well plates and after overnight glucose starvation, cells were treated with various concentrations of Eudragit blank SLNs, Eudragit Ex-4 SLNs, and Ex-4. The treated cells were extracted using 0.1 M HCl to avoid degradation of cAMP, and cAMP levels were measured in whole cell lysates by using the competitive cAMP immunoassay kit (Enzo Life Sciences) according to the manufacturer's instructions. The level of cAMP production was normalized to the amount of total protein.

2.6. Glucose-Stimulated Insulin Secretion Assay. Before the secretion experiments, the standard culture medium containing 11.1 mmol/L glucose was switched to a medium containing 5 mmol/L glucose. The insulin secretion was assayed as described previously [16]. The amount of insulin released

into the supernatant was quantified using an insulin EIA kit (Alpco Diagnostics) according to the manufacturer's instructions. The amount of insulin release was normalized to the amount of total protein.

2.7. RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). RNA isolation was performed as described previously [17], and qRT-PCR analysis was performed using SYBR master mix (Takara) using ABI 7900 HT detection system (Applied Biosystems, Carlsbad, CA, USA). The relative mRNA transcript levels were calculated according to the $2^{-\Delta CT}$ method, in which ΔCT represents the differences in threshold cycle values between the target mRNA and cyclophilin internal control. Primer sequences were as follows: 5'-GCTTTGTCAACAGCACCTTG-3' and 5'-CTCCCCACACACCAGGTAGAG-3' for insulin and 5'-GGTCTTTGGGAAGGTGAAAGAA-3' and 5'-GCCATTCTGGACCCAAA-3' for cyclophilin.

2.8. Statistical Analysis. Results were expressed as mean ± standard error of mean (SEM) of three separate experiments and were analyzed using one-way analysis of variance (ANOVA). ANOVA followed by Scheffé's multiple test was used to determine the significance of any differences among more than two groups. $P < 0.05$ was considered significant.

3. Results and Discussion

Most of the existing nanocarriers for oral delivery of Ex-4 are polymer-based nanoparticles, such as chitosan and polylactide glycolic acid (PLGA) nanoparticles [8, 18], but polymeric nanoparticles have disadvantages such as poor bioavailability and low chemical and enzyme stability [19]. A lipid-based delivery system can overcome the disadvantages of the polymeric system, and the use of such a system for the delivery of insulin has been examined [15, 20]. Few studies have examined the use of such lipid systems for oral delivery of Ex-4; however, in a recent study, Ex-4-loaded nanoparticles with a mixed lipid shell and an aqueous core containing micelles for improved hypoglycemic control have been examined [21]. In this study, Ex-4-loaded sodium cholate micelles were used as the inner aqueous phase, and this nanoparticle greatly enhanced the cellular uptake and transport of encapsulated Ex-4. In our study, Precirol, a glycerol palmitostearate, was used for the encapsulation of Ex-4 as a solid lipid (Figure 1). Precirol based SLNs showed low-particle size, high encapsulation and loading efficiency, and long-term physical stability [22]. Moreover, solid state slows down their digestion by pancreatic lipase [23] and Precirol based SLNs can be orally administered for a controlled release in the gastrointestinal tract [24]. To determine whether the Ex-4 SLNs could be an alternative to Ex-4, we examined their biological effects such as insulinotropic effects and compared them with those of native Ex-4 on the pancreatic beta cell line INS-1.

3.1. Eudragit Ex-4 SLNs Have No Cellular Toxicity. To evaluate toxicity associated with the nanomaterials, we measured the

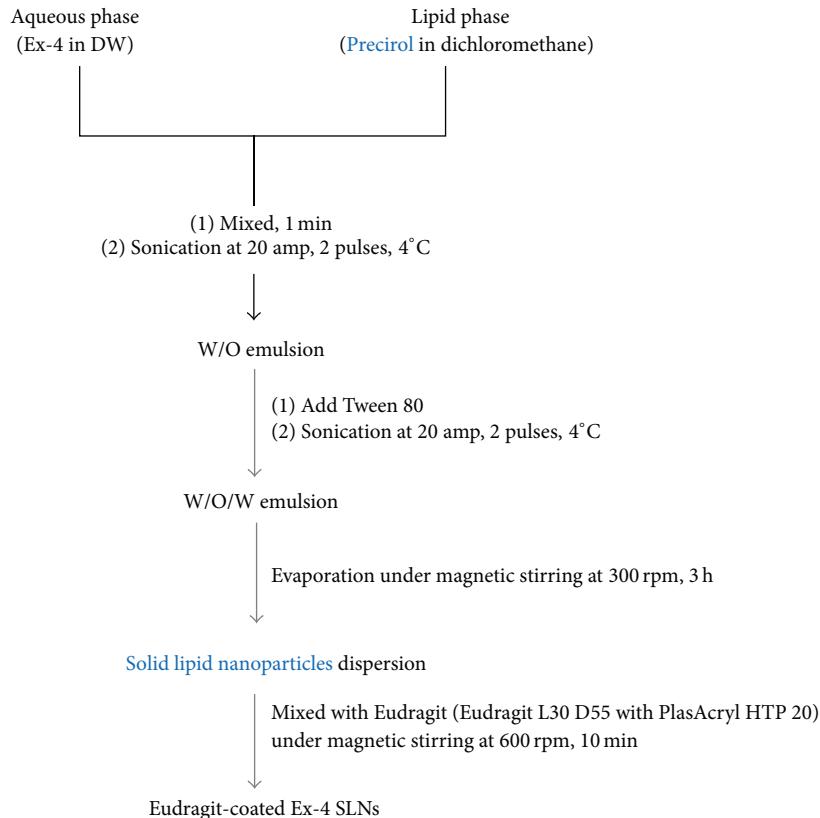


FIGURE 1: Encapsulation process of exendin-4 into solid lipid nanoparticles. The solid lipid system using Precirol was used for encapsulation of Ex-4 and nanoparticles were prepared by double emulsion (W/O/W) method.

viability of INS-1 cells incubated with different concentrations of the nanoparticles. The cells were treated with 1 to 100 nM of nanoparticles with (Eudragit Ex-4 SLNs) or without Ex-4 (Eudragit blank SLNs), and cell viability was measured using CCK assay at 24 (Figure 2(a)) and 48 h (Figure 2(b)). Treatment with the nanoparticles did not have any significant effects on cell viability, thereby confirming the safety of the carrier system (Figure 2). Compared to some polymeric nanoparticles, SLNs are generally less toxic because of the physiological and biocompatible lipids utilized in the manufacturing process [25]. Moreover, the material used for enteric coating (Eudragit L30D55), which can protect Ex-4 from the acidic environment in the stomach, itself is not cytotoxic [26]; therefore, Eudragit Ex-4 SLNs could be used as an alternative drug system *in vivo*.

3.2. Similar to Native Ex-4, Eudragit Ex-4 SLNs Increase cAMP Production. Ex-4 binds to the GLP-1 receptor (GLP-IR) coupled with Gs proteins, which stimulates adenylate cyclase and thus leads to an increase in the intracellular cAMP levels and an activation of protein kinase A (PKA) [27]. To determine whether nanoparticles impaired the ability of the Ex-4 moiety to activate the GLP-IR, we compared the potency of Eudragit Ex-4 SLNs with that of native Ex-4. We used a competitive cAMP assay to monitor ligand-dependent GLP-IR activation in INS-1 cells expressing GLP-IR. 10 nM Eudragit Ex-4 SLNs showed increase in the cAMP

levels and the levels were maintained by 100 nM treatment (Figure 3). Moreover, Göke et al. showed that RINm5F cells had maximum levels of intracellular cAMP after treatment with 10 nM Ex-4 [28]. In contrast, Eudragit blank SLNs did not alter the cAMP levels. Ex-4 seemed slightly more efficient in stimulating cAMP production than Eudragit Ex-4 SLNs; however, the increase was not significant. These results suggested that the receptor binding affinities of Eudragit Ex-4 SLNs were not different from those of Ex-4.

3.3. Eudragit Ex-4 SLNs Showed Activity Similar to Ex-4 on the Induction of Insulin mRNA Levels and Glucose-Stimulated Insulin Secretion. Physiologically, the activation of GLP-IR via Ex-4 stimulates insulin secretory capacity by augmenting the transcription of insulin gene and proinsulin synthesis and potentiates glucose-stimulated insulin secretion in pancreatic beta cells [29]. Therefore, we investigated the insulinotropic activities of Eudragit Ex-4 SLNs in INS-1 cells. Highest levels of cAMP production were observed at 10 nM; therefore, we evaluated insulin transcription levels and glucose-stimulated insulin secretion after treatment for 24 h under this condition. Compared to the vehicle, Eudragit Ex-4 SLNs increased the insulin mRNA levels, and the level of induction was similar to that of Ex-4 (Figure 4(a)). At low glucose levels (3 mM glucose), the untreated group (nontreated), Eudragit blank SLNs, Ex-4, and Eudragit Ex-4 SLNs had no effect on insulin release. However, treatment with high

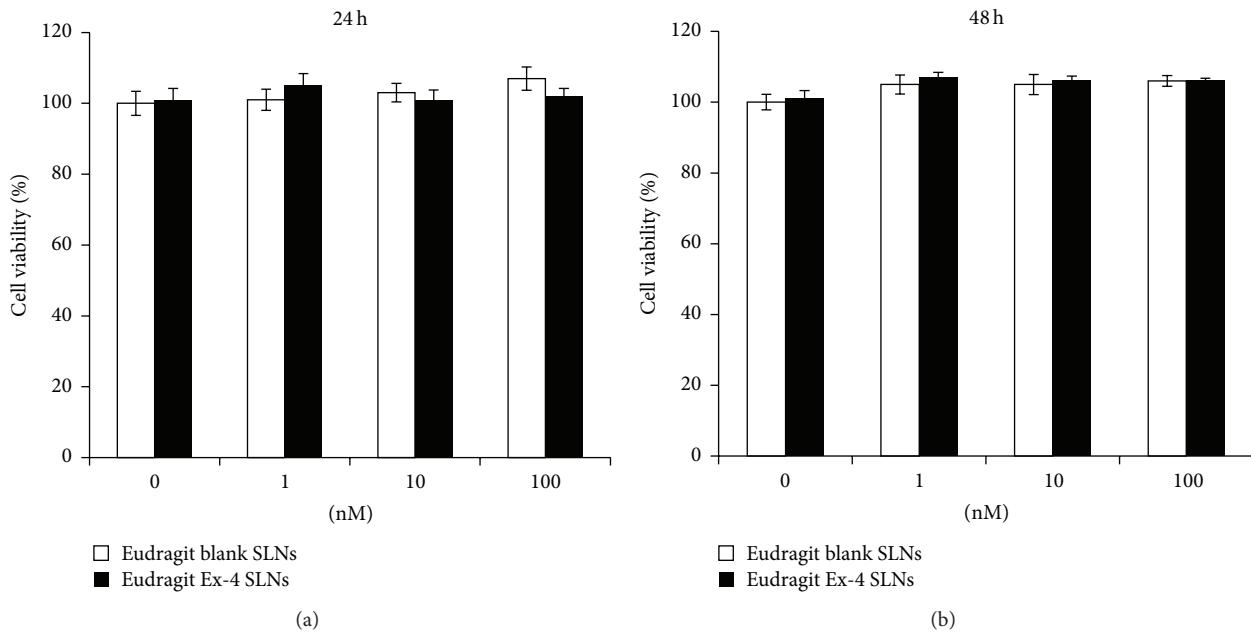


FIGURE 2: Cellular toxicity test of Eudragit exendin-4-encapsulated solid lipid nanoparticles on INS-1 cells. The number of viable INS-1 cells was determined after incubation with various concentrations of Eudragit blank solid lipid nanoparticles (SLNs) and Eudragit exendin-4 (Ex-4) SLNs for indicated times ((a) 24 h; (b) 48 h). Relative cell numbers are expressed as fold changes over nontreated cells. Data were represented as mean \pm standard error of mean (SEM; $n = 3$).

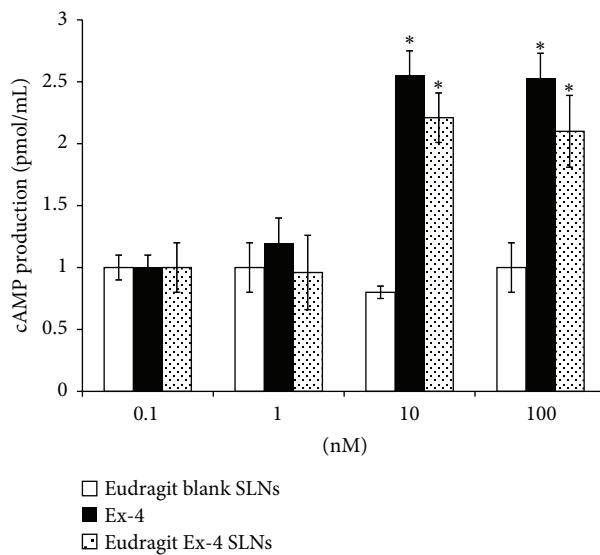


FIGURE 3: cAMP production in Eudragit exendin-4-encapsulated solid lipid nanoparticles-treated INS-1 cells. INS-1 cells were treated with different concentrations of exendin-4 (Ex-4), Eudragit blank solid lipid nanoparticles (SLNs), and Eudragit Ex-4 SLNs for 15 min and the cells were extracted using 0.1 M HCl. cAMP production was examined in the whole cell lysate using competitive cAMP immunoassay kit. Data were represented as mean \pm standard error of mean (SEM; $n = 3$). * $P < 0.05$ versus Eudragit blank SLNs.

glucose (17 mM) induced insulin secretion (nontreated), and treatment with Eudragit blank SLNs showed a similar rate of insulin secretion as that in nontreated cells. Compared to Eudragit blank SLNs, Eudragit Ex-4 SLNs significantly increased insulin secretion at high glucose concentrations. In addition, native Ex-4 increased the insulin secretion similar

to that by Eudragit Ex-4 SLNs (Figure 4(b)). These results suggest that Eudragit Ex-4 SLNs had similar effects on insulin secretion to those of native Ex-4. To determine the potential use of Eudragit Ex-4 SLNs as potent oral antidiabetic agents, the hypoglycemic effects of Eudragit Ex-4 SLNs in an animal model of diabetes will be investigated in future studies.

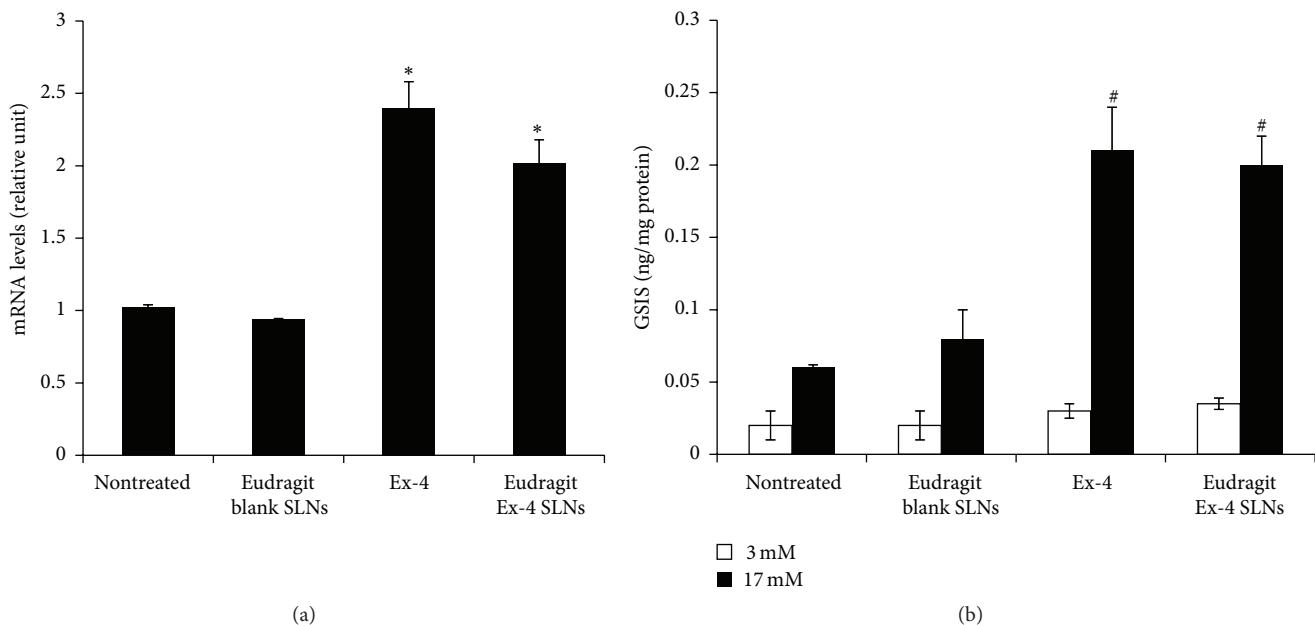


FIGURE 4: Insulinotropic activities of Eudragit exendin-4-encapsulated solid lipid nanoparticles in INS-1 cells. (a) Exendin-4 (Ex-4), Eudragit blank solid lipid nanoparticles (SLNs), and Eudragit Ex-4 SLNs were incubated for 24 h, and the insulin mRNA levels were examined using quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Cyclophilin was used as an internal control. (b) Cells were treated as described above, and after 24 h, the cells were incubated in low (3 mM) or high (17 mM) concentration of glucose. The amount of insulin released into the supernatant was quantified using a rat insulin enzyme immunoassay (EIA) kit, and the level of insulin was normalized to the total amount of protein. Data were represented as mean \pm standard error of mean (SEM; $n = 3$), * $P < 0.05$ versus Eudragit blank SLNs, # < 0.05 versus 17 mM treated Eudragit blank SLNs.

4. Conclusion

Ex-4-loaded Precirol based SLNs were prepared using a simple process based on the double emulsion technique. The Ex-4 nanoparticles had no cytotoxic effects and increased the cAMP activity similar to that induced by Ex-4 and consequently facilitated insulin secretion in response to high glucose concentrations. These *in vitro* results indicated that Eudragit Ex-4 SLNs have biological efficacies similar to those of Ex-4, and further studies about the time dependence of the biological effect of Eudragit Ex-4 SLNs *in vitro* and in animal models are required to determine the use of Eudragit Ex-4 SLNs for sustained release or oral delivery.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

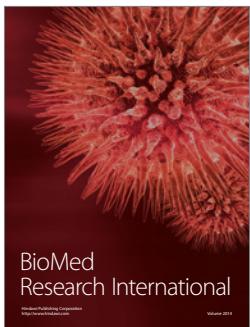
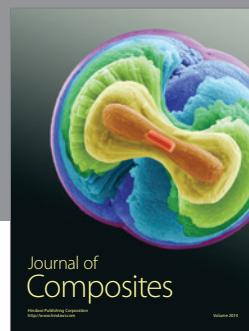
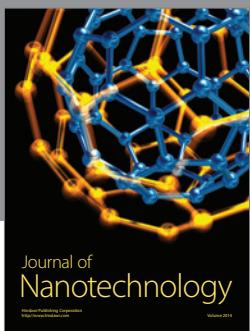
Acknowledgment

This study was supported by a grant from Korea Health Technology R&D Project through Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (Grant no. HI14C1135).

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