International Journal of Pharmaceutics 427 (2012) 417–425

Contents lists available at SciVerse ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

# Fabrication of cross-linked alginate beads using electrospraying for adenovirus delivery

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ARTICLE INFO

*Article history:*
Received 19 August 2011
Received in revised form 5 December 2011
Accepted 24 January 2012
Available online 2 February 2012

*Keywords:*
Electrospraying
Encapsulation
Adenovirus
Alginate
Controlled release

ABSTRACT

Cross-linked alginate beads containing adenovirus (Ad) were successfully fabricated using an electrospraying method to achieve the protection and release of Ad in a controlled manner. An aqueous alginate solution containing Ad was electrosprayed into an aqueous phase containing a cross-linking agent (calcium chloride) at different process variables (voltages, alginate concentrations, and flow rates). Alginate beads containing Ad were used for transduction of U343 glioma cells and the transduction efficiency of the alginate beads was measured by quantification of gene expression using a fluorescence-activated cell sorter at different time points. In vitro results of gene expression revealed that the Ad encapsulated in the alginate beads with 0.5 wt% of alginate concentration exhibited a high activity for a long period (over 7 days) and was released in a sustained manner from the alginate beads. The Ad-encapsulating alginate beads could be promising materials for local delivery of Ad at a high concentration into target sites.


## 1. Introduction

In the last few decades, adenovirus (Ad)-based vectors have attracted much attention as delivery vesicles for human gene therapy because of their high titer capability and transduction efficiency in cancer cells (Turner et al., 2007). However, their fragility against the inflammatory response and the transient nature of transgene expression have been pointed out as major obstacles for clinical applications (Aggarwal et al., 1999). Furthermore, Ad-based vectors often result in a vector-specific immune response, which significantly reduces vector uptake upon the second administration of the same vector (Beer et al., 1998; Kikuchi et al., 1999; Steel et al., 2004). For successful treatment using Ads as vectors, they should have a viral physical stability, inhibit virus aggregation under acidic conditions in a target site to reduce the vector immune response, and prolong therapeutic functions without side effects, even at a high dosage (Aggarwal et al., 1999). The advantages of Ad as a vector include (1) the relative simplicity in the manipulation of the viral genome, (2) a high gene transfer efficiency, and (3) an ability to transduce quiescent and nonquiescent cells (El-Aneed, 2004).

A variety of matrices, including avidin microspheres, antibody microbeads, and poly(D,L-lactic-co-glycolic acid) microspheres, have been used to improve the stability of Ad and the gene transfection efficiency at a target site (Beer et al., 1998; Steel et al., 2004). Although most of these matrices have showed a favorable protection effect of Ad against the host immune response, there were still shortcomings such as (1) a broad distribution of the particle size, (2) a low encapsulation efficiency of Ad, and (3) a burst release of Ad at an initial stage.

To address these issues, we fabricated Ad-encapsulating alginate beads using electrospraying for the injectable delivery of Ad at a target site using a needle. Compared to other materials, alginate is often used to fabricate a cross-linked hydrogel for the delivery of drugs, proteins, cells, and viruses due to its simple, fast, and non-toxic cross-linking procedure and control over crosslinking density as well as excellent biocompatibility (Aggarwal et al., 1999; Koch et al., 2003; Lee et al., 2008; Lim et al., 2010; Li et al., 2002; Jung et al., 2007). Electrospraying is a versatile technique to produce microspheres or beads from various materials (Bugarski et al., 1994; Goosen et al., 1997; Beer et al., 1998; Pandori et al., 2002; Huang et al., 2003; Steel et al., 2004). When an aqueous alginate solution containing Ad was electrosprayed into an aqueous solution containing calcium chloride, the alginate solution droplets were instantly cross-linked, evolving into Ad-encapsulating alginate beads.

The aim of this work is to fabricate Ad-encapsulating alginate beads using electrospraying and to optimize the process variables

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doi:10.1016/j.ijpharm.2012.01.050

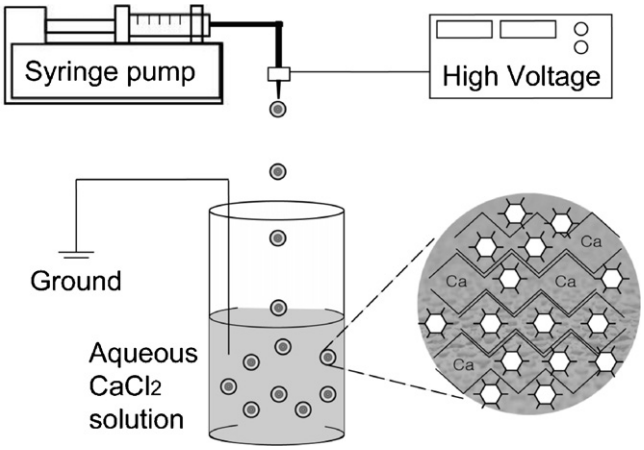


**Fig. 1.** A schematic diagram for the fabrication of Ad-encapsulating alginate beads using electrospraying.

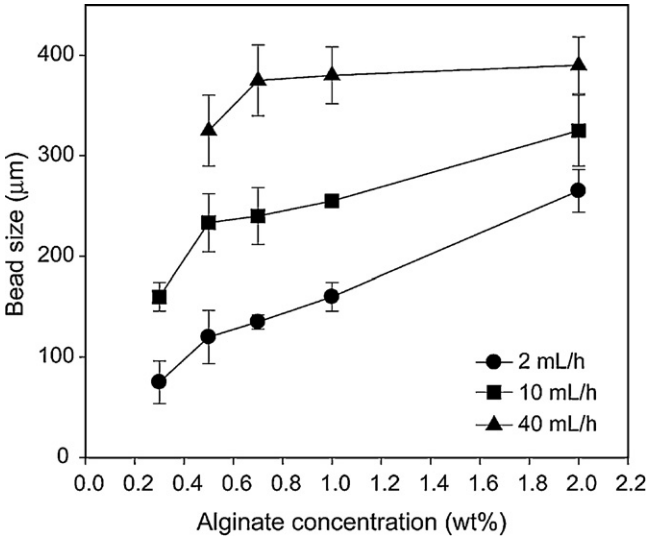


**Fig. 3.** Variations in size of alginate beads with respect to the concentration and flow rate of the alginate solution ($n = 100$).

(voltage, alginate concentrations, and flow rate), eventually to demonstrate the protection and controlled release of Ad. Specifically, we studied variations in the morphology and size of alginate beads with respect to the alginate concentration and flow rate, the effect of a high voltage on Ad activity, and the protection and release behavior of Ad in alginate beads. We believe that our strategy can be useful for the local delivery of Ad to target sites at a high concentration and subsequent enhancement of the therapeutic efficiency of Ad for cancer treatment.

## 2. Materials and methods

### 2.1. Materials

Ad encoding green fluorescence protein (GFP) gene was obtained from the Institute for Cancer Research of Yonsei University College of Medicine (Seoul, Korea) and U343 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA).

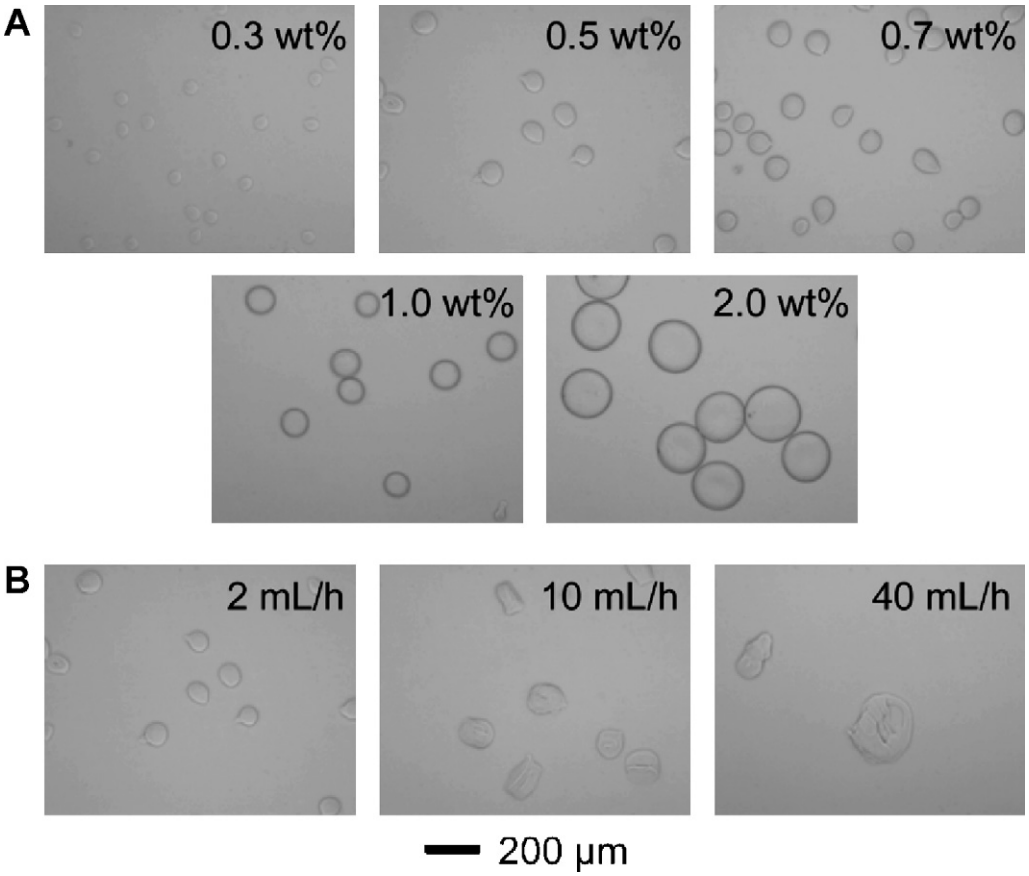


**Fig. 2.** Optical microscopy images of alginate beads prepared (A) at different alginate concentrations while flow rate was kept as 2 mL/h and (B) at different flow rates while the alginate concentration was kept as 0.5 wt%.

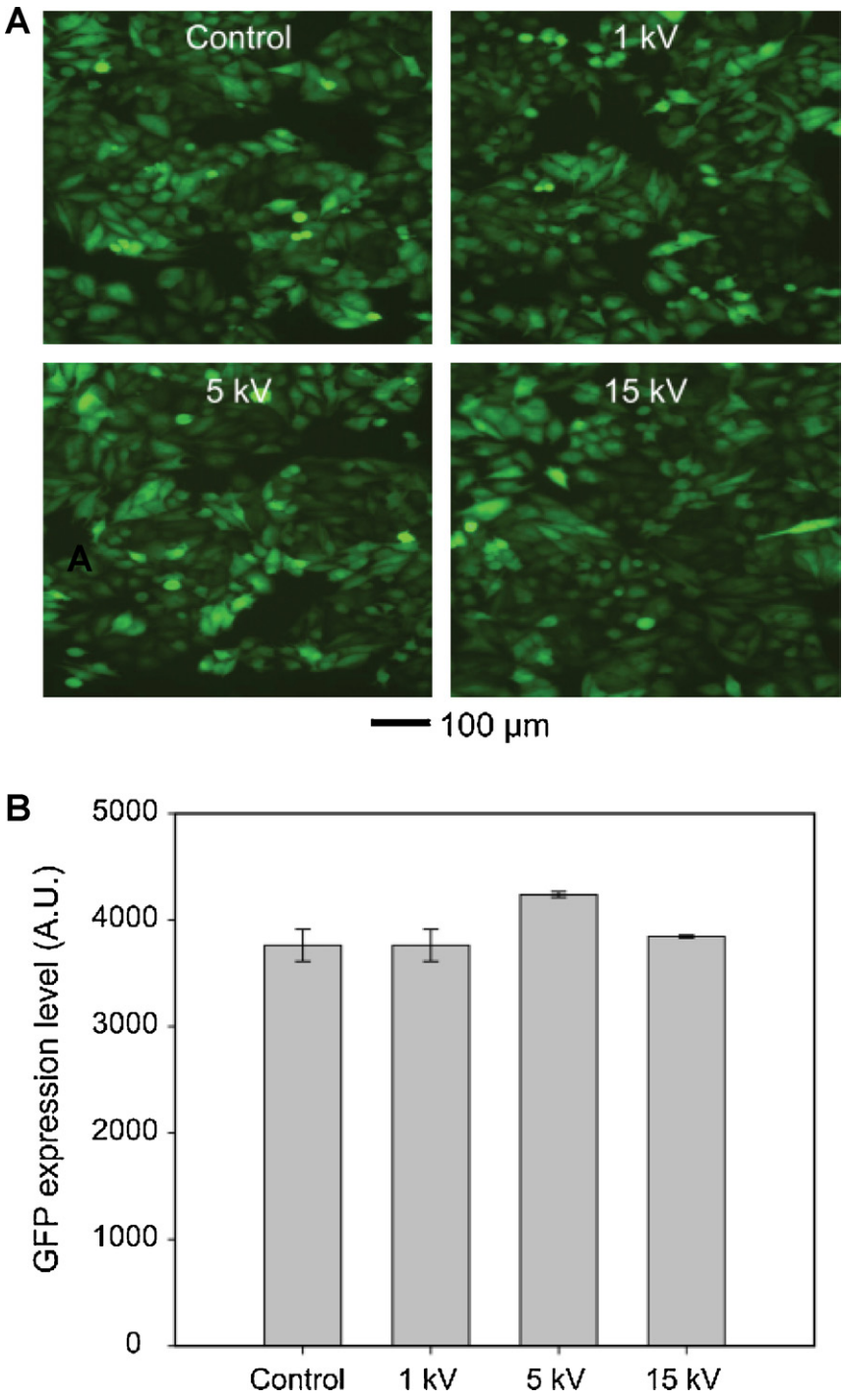


**Fig. 4.** (A) Fluorescence microscopy images of cells infected by Ad before and after electrospraying (100×) and (B) corresponding FACS results ($n = 3$).

Cell culture media were obtained from Gibco (Grand Island, NY, USA). Alginate (Sodium salt type, 100–300 cP (2 wt%) at 25 °C) and calcium chloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). An electrospraying device including a high-voltage supply, a syringe pump, a stainless-steel nozzle, and a 30-gauge syringe needle was purchased from NanoNC (NNC-ESP 200, Seoul, Korea). Cellulose tubular membrane for dialysis was purchased from Membrane Filtration Products Inc. (Cellu Sep T3, MWCO 12,000–14,000, USA). All other materials were of analytical grade and were used without further purification. All of the experiments except for the electrospraying process were conducted on a clean bench.

### 2.2. Encapsulation of Ad in alginate beads using electrospraying

U343 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin–streptomycin (100 IU/mL) at 37 °C in a humidified 5% $CO_2$ incubator.

To evaluate Ad activity during the electrospraying process, U343 cells were seeded on a 6-well plate ($1 \times 10^5$ cells/well) for 24 h before Ad transduction. Phosphate buffered saline (PBS, pH 7.4) solution containing Ad was electrosprayed into a sterilized 100 mL beaker at 1, 5, and 15 kV of applied voltage. The electrospun Ad

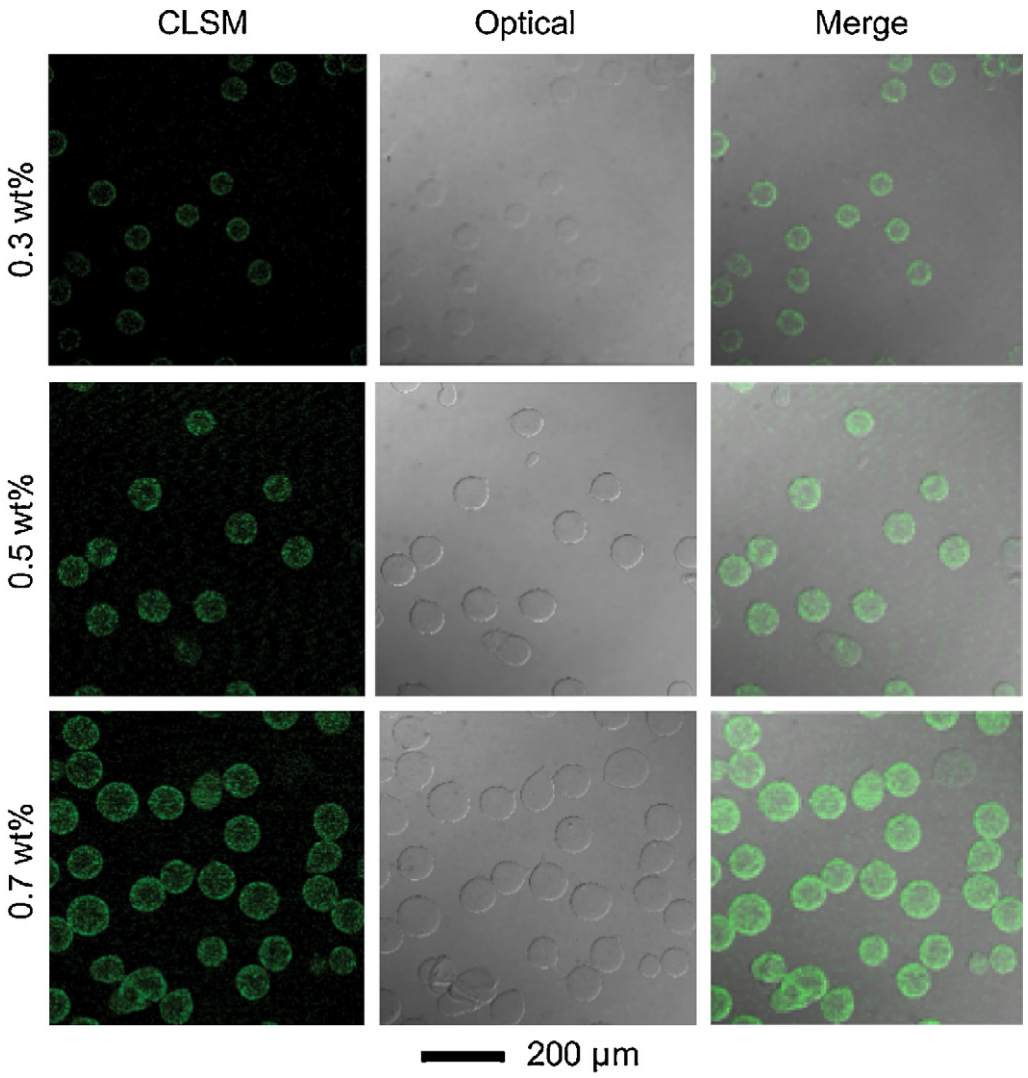


**Fig. 5.** Confocal laser scanning microscopy images of Ad (green fluorescence) encapsulated in alginate beads prepared at different alginate concentrations.

dispersion in PBS was inoculated in the U343 cells for GFP expression. The GFP expression of the Ad passed through electrospraying was observed by a fluorescence microscope (Olympus BX51, Olympus Optical, Tokyo, Japan) with a MetaMorph imaging system (Molecular Devices, USA) at 48 h. For a quantitative analysis, the cells were collected by trypsinization at 48 h and washed 3 times with PBS. The cells in final volume of 500 μL in PBS were analyzed using a fluorescence-activated cell sorter (FACS) with FACScan (Beckton-Dickinson, Sunnyvale, CA, USA). The percentages of GFP-positive cells were up to 98% in all groups.

To evaluate the effects of the process variables on the morphology of alginate beads, aqueous alginate solutions with different concentrations (0.3, 0.5, 0.7, 1.0, and 2.0 wt%) were electrosprayed into 20 mL of an aqueous calcium chloride solution (30 mM) through a 30-gauge needle under various voltages (1, 4, 7, 11, and 15 kV), where the distance between the needle tip and the top surface of the calcium chloride solution in the beaker was set to 10 cm. The flow rate of the alginate solution varied at 2, 10, and 40 mL/h. The size and morphology of the resulting alginate beads were observed by an optical microscope (Olympus CKX41, Olympus Corporation, Tokyo, Japan). The average size and standard deviation of the beads was calculated from the optical microscopy images by analyzing at least 100 beads for each sample using ImageJ software (National Institutes of Health, USA).

To visualize the presence of Ad in the alginate beads, we used $1 \times 10^9$ viral particles (VP)/mL of replication-incompetent Ad (Ad-ΔE1/IX-GFP) genetically labeled with GFP on the pIX of the viral capsid. After electrospraying the alginate solution (0.3, 0.5, and 0.7 wt% of alginate concentration) containing the Ad, confocal laser scanning microscopy (LSM 510 META, Carl Zeiss, Oberkochen, Germany) was used to observe the Ad within the alginate beads, where fluorescence was detected by a 505–530 nm bypass filter using an argon laser of 488 nm.

### 2.3. In vitro delivery of Ad from alginate beads to cancer cells

For release test, an aqueous alginate solution (0.3, 0.5, 0.7, and 1.0 wt%) mixed with Ad carrying GFP gene ($3.14 \times 10^{12}$ VP/mL) was electrosprayed at 2 mL/h of flow rate and 11 kV of applied voltage to prepare alginate beads with different alginate concentrations. The resulting alginate beads were collected by sieving (45 μm of mesh size), washed with PBS, and dialyzed using PBS overnight (MWCO 12,000–14,000) to remove unencapsulated Ad and calcium chloride.

To test viral infection with the Ad released from the alginate beads (0.5 wt% of alginate concentration), U343 cells ($1 \times 10^5$ cells/well) were seeded in a 6-well plate before Ad transduction. After 24 h, 200 μL of the dispersion containing

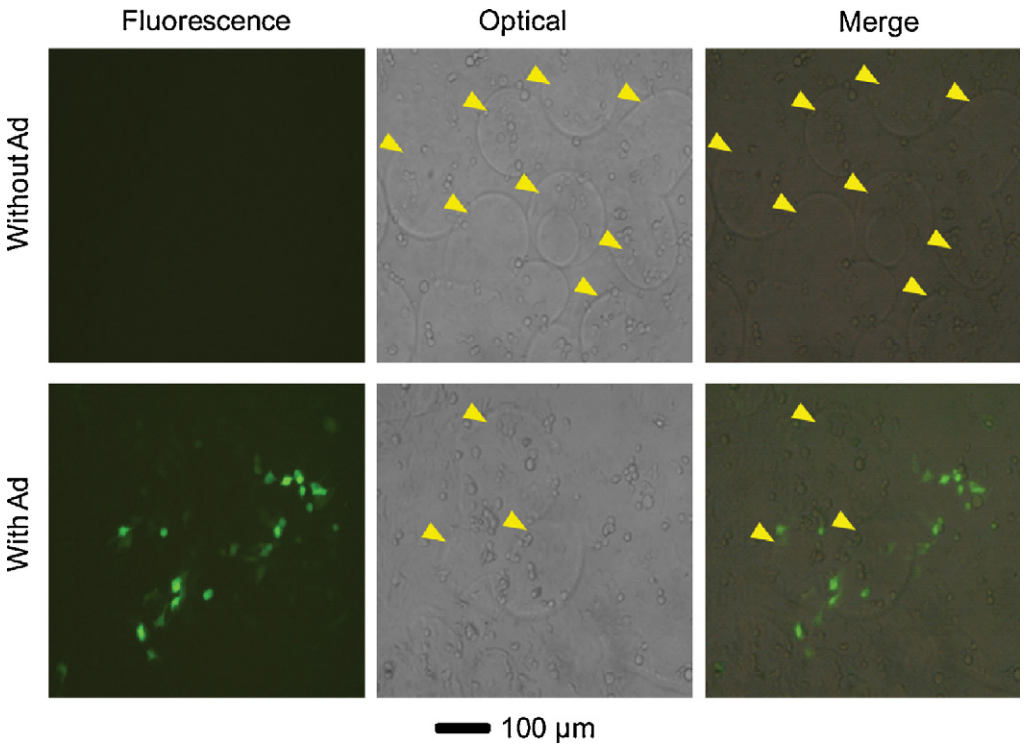


**Fig. 6.** Fluorescence, optical, and merged images of cells infected by Ad released from alginate beads prepared at 0.5 wt% of alginate concentration.

approximately 2500 alginate beads was directly placed into each well, where alginate beads without Ad served as a control. Fluorescence microscopy images for GFP expression were taken at 48 h post-infection.

To evaluate the effects of alginate concentration on the release behavior and activity of Ad, the alginate bead dispersion (2 mL) was poured into 15 mL Falcon tubes and stored in an incubator at 37 °C. At 1, 3, 5, and 7 days, the released medium (1.5 mL) was collected for GFP transduction and the same volume of fresh PBS was added. The released medium containing Ad was placed into the U343 cells and then 3 mL of DMEM with 5% FBS was added. At 48 h post-infection, the cells were observed by fluorescence microscopy and the GFP expression level was quantitatively analyzed using FACS analysis. For quantification of the Ad remained in the alginate beads, 200 μL of a dissolution solution (55 mM sodium citrate, 0.15 M sodium chloride, and 30 mM EDTA) was added into the alginate bead dispersion in the Falcon tube to dissolve the cross-linked alginate beads prior to GFP transduction analysis.

For the statistics, the experimental results were expressed as the means ± standard deviation (s.d.). Statistical comparisons were evaluated by ANOVA and statistical significance was accepted at $p < 0.05$.

## 3. Results and discussion

### 3.1. Characterization of alginate beads

As shown in Fig. 1, Ad-encapsulating alginate beads were prepared using electrospraying in sterile conditions. Under a high applied voltage, an alginate solution containing Ad was dropped into an aqueous solution containing calcium chloride (cross-linker for alginate), instantly forming cross-linked beads due to the rapid ionotropic reaction between alginate and calcium cations (Strand et al., 2002; Zhang et al., 2008).

To evaluate the effect of an applied voltage, alginate solution (1.0 wt%) without Ad was electrosprayed under different applied voltages (1, 4, 7, 11, and 15 kV) at a constant flow rate (2 mL/h). The average size of alginate beads was approximately 3 mm in diameter at the low applied voltages (1 and 4 kV) and tended to decrease with the increase in the applied voltage. At 11 kV, the average size of alginate beads was notably reduced to several hundred micrometers. No significant reduction in the average size of alginate beads was observed above 11 kV. Therefore, the applied voltage was kept at 11 kV for the rest of the experiments.

Fig. 2 shows the optical microscopy images of the alginate beads prepared at different alginate concentrations and flow rates. The average size and standard deviation of the alginate beads are presented in Table S1 of Supplementary Materials. The majority of the alginate beads prepared at less than 0.7 wt% of alginate concentration were not perfectly spherical in shape. In addition, alginate beads prepared at over 10 mL/h of flow rate exhibited irregular shapes. These results suggest that a higher alginate concentration and a lower flow rate are favorable conditions for the spherical morphology of alginate beads.

Fig. 3 shows the variation in the average size of alginate beads at the different concentrations and flow rates of alginate solution. The low alginate concentration and flow rate led to reduction in the average size of alginate beads due to the low viscosity of the alginate solution (Choi et al., 2009) and the slow formation of droplets at the end of the needle during electrospraying. At 40 mL/h of flow rate, alginate solution with 0.3 wt% of alginate concentration formed a fibrous morphology due to the very low viscosity and exceedingly high flow rate. In all experimental ranges, the presence of Ad did not significantly affect the size of the alginate beads. Although few studies have addressed the optimum size for injection into a human body, alginate beads with less than 150 μm in size may be favorable for preventing occlusion during injection using a 26-gauge needle. In our approach, alginate beads with less than 150 μm in size could be obtained when the alginate concentration and flow rate were less than 1.0 wt% and 2 mL/h, respectively.

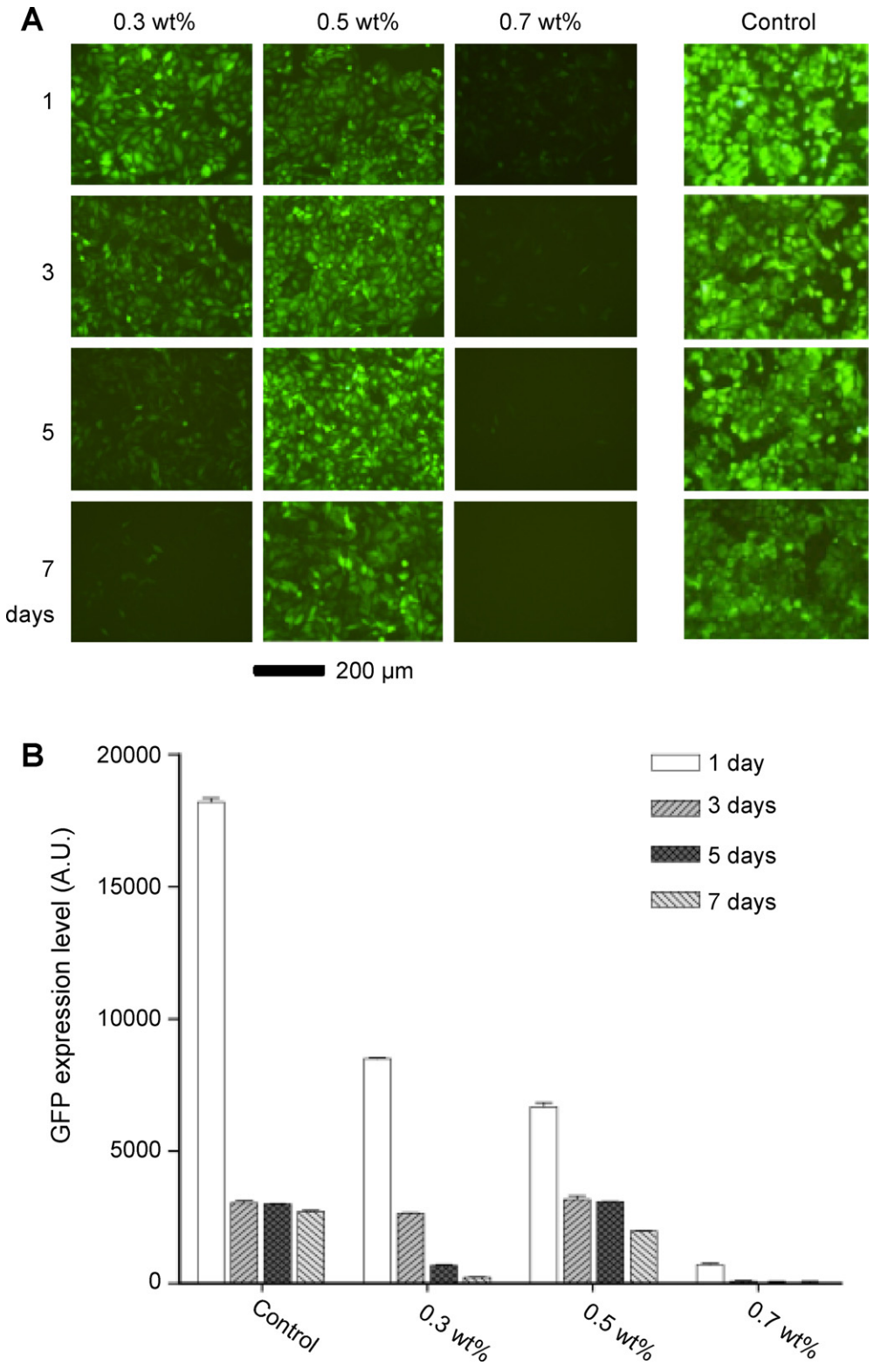


**Fig. 7.** (A) Representative fluorescence microscopy images of GFP expressed cells and (B) corresponding FACS results after transduction with the Ad released from alginate beads with different concentrations (0.3, 0.5, and 0.7 wt%) at 1, 3, 5, and 7 days ($n = 3$).

### 3.2. Effect of high voltage on Ad activity

To verify the effect of high voltage on Ad activity, PBS containing Ad ($3.14 \times 10^{12}$ VP/mL) was electrosprayed into PBS at 1, 5, and 15 kV and the transduction efficiency of Ad to cancer cells was examined. As shown in Fig. 4, there was no quantitative difference in the GFP expression levels before and after electrospraying even at 15 kV, suggesting that Ad was not affected by exposure to the high voltage because the applied voltages had low electric potential on Ad and the exposing time was quite short (Park et al., 2010a,b).

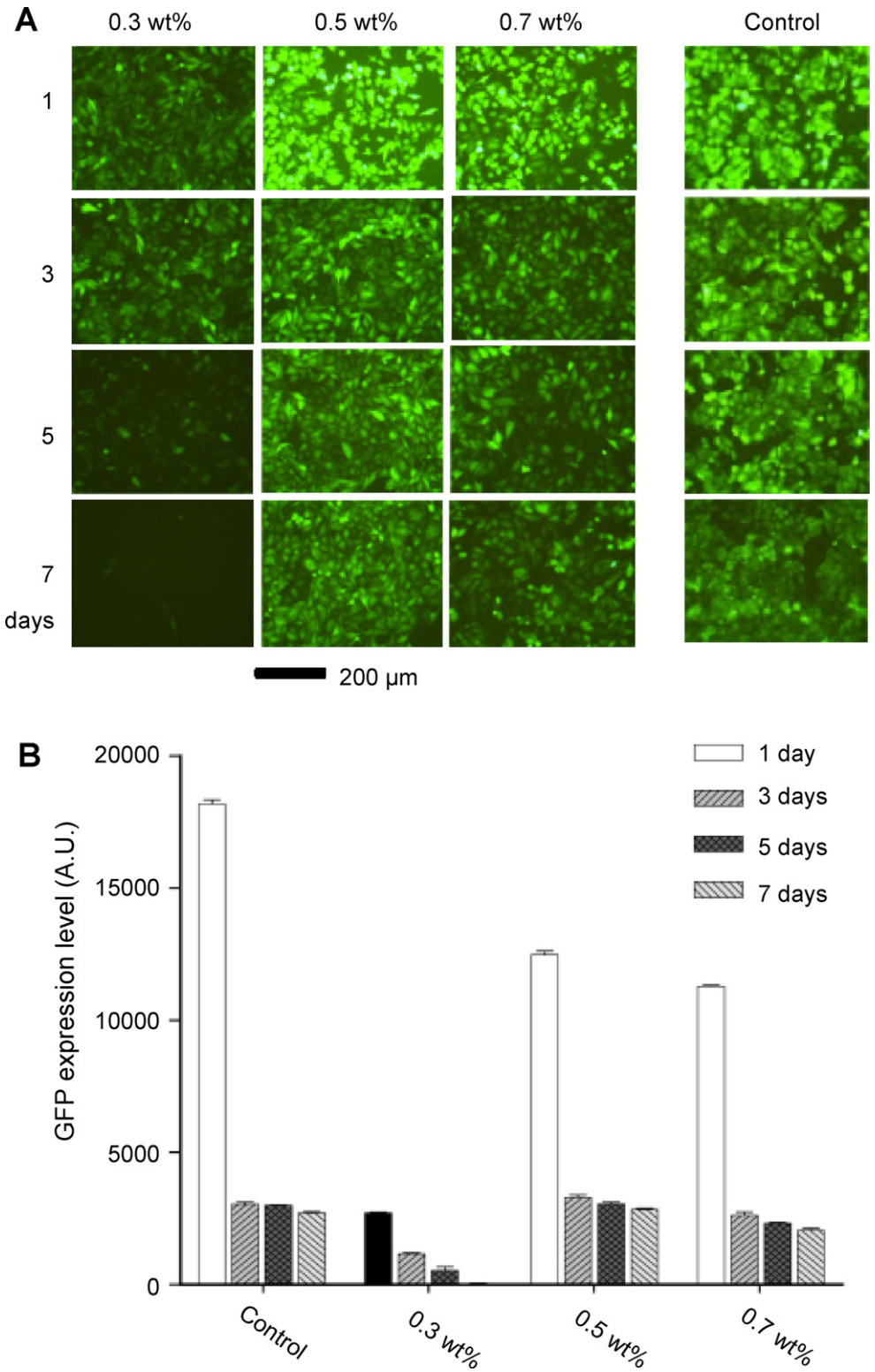


**Fig. 8.** (A) Representative fluorescence microscopy images of GFP expressed cells and (B) corresponding FACS results after transduction with the Ad remained in alginate beads with different concentrations (0.3, 0.5, and 0.7 wt%) at 1, 3, 5, and 7 days ($n = 3$).

### 3.3. Encapsulation of Ad in alginate beads

To visualize Ad encapsulated in the alginate beads, we used $1 \times 10^9$ VP of replication-incompetent Ad (ΔE1/IX-GFP) genetically labeled with GFP on the pIX of a viral capsid. Alginate solutions with different concentrations (0.3, 0.5, and 0.7 wt%) containing Ad were electrosprayed at 11 kV of an applied voltage of and 2 mL/h of flow rate. Fig. 5 shows confocal laser scanning microscopy (CLSM) and optical microscopy (OM) images of the Ad-encapsulating alginate beads. It was found that alginate beads with a high alginate concentration exhibited strong fluorescence intensities, suggesting high encapsulation efficiency due to the dense alginate network

(George and Abraham, 2007; Serp et al., 2002). Beside fluorescence intensity in the alginate beads, both the quantitative analysis using Polymerase Chain Reaction (PCR) method and the GFP expression level of the unencapsulated Ad showed the corresponding results with Fig. 5 (Supplementary Materials, Fig. S1 and S2).

### 3.4. In vitro transduction efficiency of Ad in alginate beads

To confirm the viral transduction with the released Ad, alginate beads (0.5 wt% of alginate concentration) containing Ad were placed onto a confluent layer of cancer cells (U343), while alginate beads without Ad served as a control. As shown in Fig. 6, the Ad released from the alginate beads was expressed in the cancer cells at the position of the beads, suggesting the gene expression capability of the released Ad and the potential of target specific release.

To evaluate the effects of alginate concentration on the behavior of Ad, Ad-encapsulating alginate beads with different alginate concentrations were analyzed using a fluorescence microscope and FACS at 1, 3, 5, and 7 days. It was found that the concentration of alginate solution significantly affected the encapsulation efficiency. Although a high concentration of alginate solution improved the encapsulation efficiency, the alginate beads with 1.0 wt% of alginate concentration did not release Ad due to their dense cross-linked network (Supplementary Materials, Fig. S3). Therefore, the concentration of alginate solution was limited to below 1 wt%. Fig. 7 shows the release behaviors of Ad from alginate beads with different alginate concentrations (0.3, 0.5, and 0.7 wt%) and the corresponding quantitative FACS results. A significant amount of Ad was released from the alginate beads with 0.3 wt% of alginate concentration at the initial stage due to the loose alginate network, after which the expression level rapidly decreased over time. The alginate beads with 0.7 wt% of alginate concentration released only a small amount of Ad due to the high degree of cross-linking (Martinsen et al., 1989; Smidsrod et al., 1990). In contrast, alginate beads with 0.5 wt% of alginate concentration released Ad in a sustained manner for 7 days.

In addition to the sustained release of Ad, it is worth pointing out that the Ad remained in the alginate beads with 0.5 wt% of alginate concentration exhibited a high activity for 7 days (Fig. 8). The activity of Ad remained in the alginate beads with 0.5 wt% of alginate solution was comparable to the control value and significantly higher than that of Ad remained in the alginate beads with 0.3 wt% of alginate concentration ($p < 0.05$), suggesting that the alginate beads exerted a protective effect from the outer environment and possibly extended the release period of Ad. Taken together, the alginate beads with a low alginate concentration have critical problems such as a burst release of Ad at the initial stage and a low activity of the Ad remained in the beads. In contrast, although alginate beads with a high alginate concentration showed a high activity of the remained Ad due to an enhanced protective effect, the amount of the released Ad was too low for effective treatment of cancer cells. Therefore, we determined that the alginate beads with the 0.5 wt% of alginate concentration had superior performance in terms of both the protective effect and the release behavior of Ad.

## 4. Conclusions

We have successfully demonstrated the encapsulation of Ad in alginate beads using electrospraying. The morphology and size of the alginate beads were dependent on the applied voltage, flow rate, and concentration of alginate solution. We demonstrated that alginate beads with 0.5 wt% of alginate concentration could encapsulate a large amount of Ad with a high activity and release Ad in a sustained manner for 7 days. The Ad released from the alginate beads exhibited high transduction efficiency in cancer cells. Our approach allows for an extended biological activity of Ad and a long-term therapeutic effect by controlled release of Ad from alginate beads. We believe that Ad-encapsulating alginate beads can overcome the limitation on liver accumulation and immune response, and offer high concentration of Ad at target site via local administration. Our next goal will be focused on an in vivo demonstration for cancer treatment.

## Acknowledgements

This work was financially supported by a Korea Science and Engineering Foundation (KOSEF) grant funded by the Korean Government (MOST) (Nos. R11-2007-050-00000-0 and R01-2007-000-10353-0), the Nano R&D program through the Korea Science and Engineering Foundation funded by the Ministry of Education, Science and Technology (2011-0019176), the Ministry of Knowledge Economy (10030051), the Korea Science and Engineering Foundation (2010-0029220, 2009K001644), Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0023064), and a grant of the Korea Healthcare technology R&D Project, Ministry of Health & Welfare, Republic of Korea (A110416).

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2012.01.050.

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