

Large-Scale Production of Ba^{2+}–Alginate-Coated Vesicles of Carbon Nanofibers for DNA-Interactive Pollutant Elimination

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Ba^{2+}–alginate coated vesicles (Ba^{2+}–ALG) containing highly dispersed carbon nanofibers (CNFs) were successfully produced for the first time using an encapsulation technique. These Ba^{2+}–ALG/CNFs composite vesicles showed high capabilities in trapping DNA-interactive types of chemicals. For example, 10.0 mL of the vesicles took 0.43 μmol of ethidium ions up from contaminated water within 8 min. Biocompatibility experiments performed in vitro and in vivo provided promising results, suggesting potential applications in in-situ environmental remediation. Kilogram quantities of the Ba^{2+}–ALG/CNFs composite vesicles can be produced within a few hours.

DNA-interactive chemicals, such as ethidium ions, are capable of interacting with DNA with high affinities. This type of chemical is responsible for causing DNA damage and/or frame-shift mutagenesis induction, also known as the key-initial processes in carcinogenesis.[1–3] We demonstrated in our previous studies[4–7] that materials having DNA as functional sites, such as Ba^{2+}–alginate coated vesicles containing double-stranded DNA (purified from salmon milt), are capable of trapping this type of chemical from contaminated waters. DNA leakage, which results in performance degradation and a limited lifetime of the functional materials, however, has been a major drawback of these materials.[8]

In this study, we use carbon nanofibers (CNFs) as the functional sites for trapping DNA-interactive chemicals. Adsorbents with high capabilities were obtained successfully by encapsulating the highly dispersed CNFs in Ba^{2+}–alginate coated vesicles. In recent years, carbon nanomaterials, especially the so-called carbon nanotubes (CNTs), discovered first by Iijima,[9,10] have attracted great attention due to their unusual morphologies. An analytical chemical study carried out by Cai and co-workers[11] demonstrated that multi-walled CNTs (MWCNTs) can trap some aromatic chemicals, such as bisphenol A, 4-*n*-nonylphone, and 4-*tert*-octylphenol from contaminated water by passing the contaminated water through a cartridge filled with MWCNTs. The targeted species trapping was achieved by the hexagonally arrayed carbon atoms of the graphite sheets of the external faces of the MWCNTs.[11,12]

CNFs are built up also by hexagonally arrayed carbon atoms with the 002 planes being piled up along the direction of the fiber axis. These graphite platelets stacked in a perfectly arranged conformation resulted in generating a unique system comprising entirely of slit-shaped wall-nanopores.[13] If these wall-nanopores can be also activized for trapping DNA-interactive chemicals, higher elimination efficiencies than can be achieved with MWCNTs can be obtained.

Experimental

Dispersing CNFs Using Alginate. Sodium alginate (viscosity and pH at 20 °C were 300–400 cP and 6.0–8.0, respectively, for a 20.0-mg/mL aqueous solution), the dispersing agent used throughout this study, was obtained from Wako Chemical Industries (Osaka, Japan). Sodium alginate (Na^+–ALG) was dissolved in deionized water to prepare the aqueous Na^+–ALG solutions. CNFs (diameter, 50–250 nm; length, 2–15 μm; purity > 90%; those produced in the laboratory were based on the chemical vapour deposition;[14] Figs. 1A and B show the SEM and TEM images) were added to the aqueous Na^+–ALG solutions and were well-mixed by a combination of high-shear mixing and sufficient ultra-sonication. The aqueous Na^+–ALG/CNFs colloidal solutions were centrifuged at 4000 rpm for 30 min. A very small amount of black precipitate from the aqueous solution was observed (which was removed from the aqueous colloidal solution).

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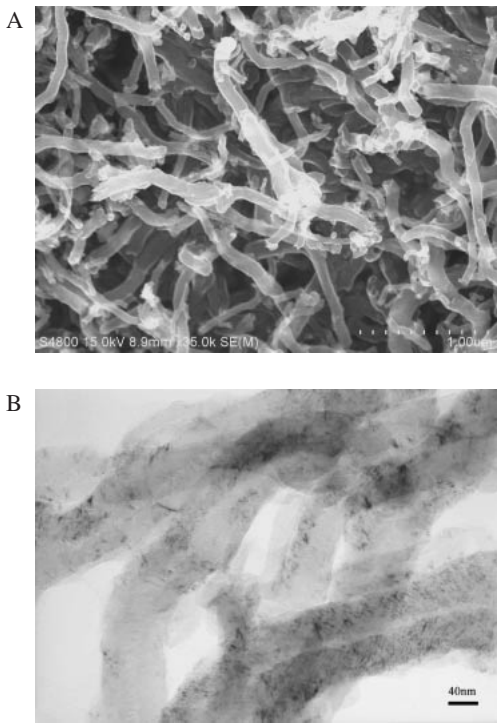

Fig. 1. SEM image (Fig. 1A) and TEM image (Fig. 1B) of the synthesized/purified carbon nanofibers (CNFs). SEM (Hitachi S4800) was operated at 15 kV; while TEM (Hitachi H-800) was operated at 200 kV. Platelet CNFs in which the 002 planes are piled up along the fiber axis direction together with the herringbone CNFs in which the 002 planes are distrusted on the two sides at an angle with respect towards the fiber axis were observed.

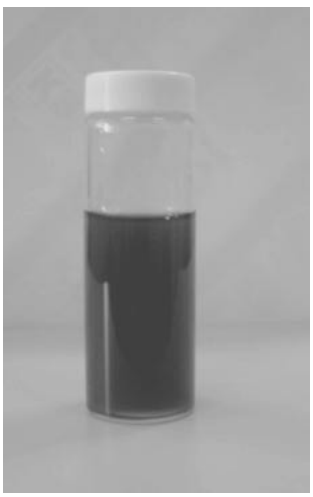

Fig. 2. A photograph of a 100-mL glass-vial containing CNFs being highly dispersed in the Na^+–ALG aqueous solution. Concentrations of CNFs and Na^+–ALG were 0.5 mg/mL and 20.0 mg/mL, respectively.

Figure 2 shows a photograph of a 100-mL glass vial containing the aqueous Na^+–ALG/CNFs colloids. Concentrations for CNFs and Na^+–ALG were 0.50 mg/mL and 20.0 mg/mL, respectively. No precipitation was observed from this aqueous colloidal solution during a three-week observation period. Aqueous Na^+–ALG/CNFs colloids with a high uniformity were obtainable up to a concentration of 1.0 mg/mL for CNFs using the 20.0 mg/mL aqueous Na^+–ALG solution as the dispersing solution. The uniformity of the aqueous Na^+–ALG/CNFs colloids was measured by calculating the linearity of the calibration curve for CNFs in the Na^+–ALG/CNFs colloidal solutions using UV–vis at 260 nm as the detection. Zeta potentials of the aqueous Na^+–ALG/CNFs colloids were measured using an electrophoretic light scattering spectrophotometer (ELS-8000, Otsuka Electronics, Osaka, Japan). FT-IR spectra of Na^+–ALG in the aqueous Na^+–ALG/CNFs colloids were measured using a FT/IR-460 (Jasco, Tokyo, Japan).

Biocompatibility Tests. Normal human fibroblasts (HF) were used as the typical cells (obtained from BioWhitteker Inc.) to perform the in vitro experiments. A MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-teraazolium] cell proliferation assay kit was purchased from Promega. Dulbecco's modified Eagle's minimal essential medium (D-MEM), L-glutamine, and fetal bovine serum (FBS) were purchased from Sigma. The aqueous Na^+–ALG/CNFs colloidal solution containing 1.0 mg/mL of CNFs and 20.0 mg/mL of Na^+–ALG was diluted to 1/10, 1/100, and 1/1000 with D-MEM containing 5.0% FBS and 50 μg/mL kanamycin. An aqueous solution containing Na^+–ALG alone (20.0 mg/mL; the control vehicle) was also diluted to the same degree with the same medium as for Na^+–ALG/CNFs. HF were seeded in 96-multiwell plates at 2×10^3 cell/well and maintained in 200 μL D-MEM containing 10% FBS and 50 μg/mL kanamycin for 3 days at 37 °C. The medium was replaced with the medium containing the Na^+–ALG/CNFs or the control vehicle and were further incubated for 1–7 days at 37 °C. After the incubation, cell growths were evaluated by MTS assay. For the MTS assay, the medium was substituted for 100 μL of Eagle's minimal essential medium (without Phenol Red) containing 333 μg/mL MTS and 25 μM phenazine methosulfate solution. After incubation for 2 h at 37 °C, the absorbance at 485 nm of each well was measured. The cell growth was calculated from the value of A_{485} at 2 h after MTS processing.

Eight 8-week-old Jcl:SD male rats (purchased from Clea Japan, Inc.) were used to perform the in vivo experiments. These rats were quarantined and acclimatized for six days. One was used without any administration while the others were single dosed orally using a stomach tube. The dose values were 10 mg/kg for CNFs and 200 mg/kg for Na^+–ALG of the body weight. Gross observations and body weights were recorded weekly. During the term of observation, animals were starved for 16 h, anesthetized, and then blood and serum samples were collected. Necropsy was also performed for observing changes in the glandular stomach.

Encapsulating CNFs. An IER-20® system (Inotech, Dottikon, Switzerland) was used for encapsulating CNFs to form Ba^{2+}–alginate coated vesicles. The IER-20® system consisted of a syringe and a pump, a pulsation chamber, a vibration system, a nozzle, an electrode, an ultra-sonication vibration system along with an electrostatic supply system, and an O-ring-shaped electrode. The aqueous Na^+–ALG/CNFs colloids were forced into the pulsation chamber using the syringe pump. These aqueous colloids were then passed through the precisely drilled sapphire-nozzle (nozzle size, 300 μm) and were separated into droplets of equal size on exiting the nozzle. These droplets passed through the electrostatic field between the nozzle and the ring electrode and acquired electrostatic charges on their surfaces. Electrostatic repulsion forces dispersed the droplets as they fell in to the hardening solution, i.e., the aqueous solution containing 100 mM of barium chloride. The resultant vesicles were rinsed thoroughly

with deionized water using a 100 μm mesh sieve. Reference vesicles (without containing CNFs) were also prepared under these identical experimental conditions.

Adsorptive Capability Studies. Three 45-mL conical-bottomed tubes, each containing 10.0 mL of the reference vesicles, the vesicles containing highly dispersed CNFs, and the vesicles containing low-dispersed CNFs, were held vertically using a tube-stand, 15.0 mL of a 30.0 μM ethidium bromide aqueous solution were then added to each of the tubes. At about ten minutes after mixing the ethidium solution with the vesicles, approximately 3 mL of the bulk-phase solutions were collected and measured using a UV–vis spectrometer (Jasco UV-550).

Results and Discussion

Insights in to the Dispersion Mechanism. A characteristic absorption band derived from the CNFs is seen at around 260 nm for the aqueous Na^+–ALG/CNFs colloidal solutions. The linearity, r^2, of the calibration curve for CNFs in the aqueous Na^+–ALG/CNFs colloidal solutions at 260 nm was found to be better than 0.9986, indicating a high uniformity of the aqueous Na^+–ALG/CNFs colloidal solutions. Samples used for deriving the calibration curve were the aqueous Na^+–ALG solutions containing 0, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL of the CNFs with the concentration of Na^+–ALG in each sample being fixed at 20.0 mg/mL. The stability of the aqueous Na^+–ALG/CNFs colloids was measured by calculating the concentration of the CNFs versus the sediment time, similar to the method reported by Jiang and co-workers.[15] Changes in the CNF concentrations were found to be smaller than 0.4% over three-weeks at room temperature.

The zeta potentials of the Na^+–ALG/CNFs colloids were measured. Zeta potential values, ζ, were calculated from the particle velocities based on the Helmholtz–Smoluchowski equation ($\zeta = 4\pi\mu\eta/D$, where, μ, η, and D are the electrophoretic mobility, viscosity, and the dielectric constant of the liquid in the boundary layer, respectively).[16] As can be seen from the ζ vs pH plots (Fig. 3), the maximum ζ value of the Na^+–ALG/CNFs colloids was −58.03 mV. This again indicates a high stability (repulsion) of the Na^+–ALG/CNFs colloids. The ζ vs pH plots (Fig. 3) for the Na^+–ALG/CNFs colloids and that for the aqueous solution containing Na^+–ALG alone were virtually identical, indicating the zeta potentials of the Na^+–ALG/CNFs colloids are governed by alginate ions. A decrease in the absolute value of ζ is seen for both samples as the pH decreased (pH of the samples were adjusted using 1.0 mol L^{-1} HCl) for all pH value studies. This implies that alginate attached tightly to the CNFs throughout the entire pH range. Note the Na^+–ALG/CNFs colloids condensed as the pH reached smaller values (2.99 and 1.51).

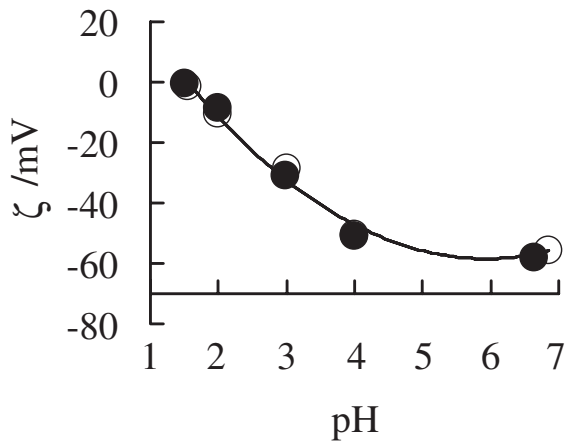

Fig. 3. Zeta potential (ζ) of the aqueous Na^+–ALG/CNFs colloids (●, 0.50 mg/mL for CNFs and 20.0 mg/mL for Na^+–ALG) and ζ of the aqueous solution containing Na^+–ALG alone (○, 20.0 mg/mL of Na^+–ALG) versus pH.

Alginate is a linear, water-soluble 1,4-linked copolymer of β-D-mannuronate (M) and/or α-L-guluronate (G). M and G can be arranged in homopolymeric [poly(β-D-mannosyluronate, M–M–M)] and poly(α-L-gulosyluronate, G–G–G) or heteropolymeric (M–G–M) blocks,[17,18] as illustrated in Fig. 4. FT-IR spectra of alginate alone (the reference sample) and the Na^+–ALG/CNFs colloids were measured, as shown in Fig. 5. The typical characteristic bands found for the reference

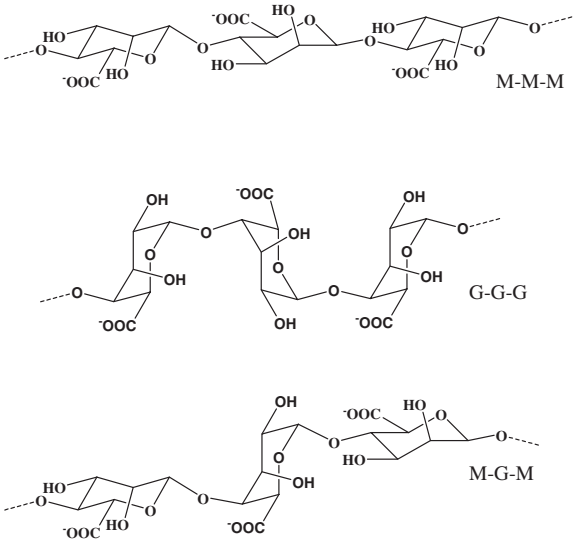

Fig. 4. Three possible molecular structures of alginate. The homopolymeric M–M–M- and G–G–G- and M–G–M-linked alginate.

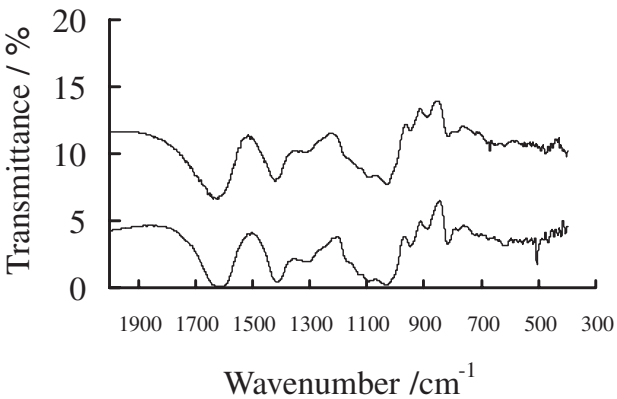

Fig. 5. FT-IR spectra of Na^+–ALG (upper-trace) and that of Na^+–ALG/CNFs colloids (lower-trace) in the solid state using the potassium bromide (KBr) pellet method.

sample (without containing CNFs) were (i) 1627.63 and 1419.35 cm^{-1} (carboxylate), (ii) 947.84 cm^{-1} (α 1 $\rightarrow$ 4 linkage), and (iii) 890.95 cm^{-1} (α-L-gulopyranuronic ring), respectively. While for Na^+–ALG/CNFs, they were (i) 1613.16 and 1413.57 cm^{-1}, (ii) 943.98 cm^{-1}, and (iii) 887.8 cm^{-1}, respectively. These shifts (decrease) in the alginate characteristic bands again indicate alginate being attached tightly to the CNFs. The characteristic band of the sugar backbones increased from 811.88 cm^{-1} in the reference sample (alginate alone) to 814.77 cm^{-1} in the Na^+–ALG/CNFs, suggesting the attaching of alginate to the CNFs is governed by their sugar backbones.

From these experimental observations, we suggest that complexes, i.e., alginate/CNFs, were formed in the aqueous Na^+–ALG/CNFs colloidal solutions. This can be attributed to the hydrophobic interactions occurring between the sugar backbones of alginate and the external surfaces of CNFs. The negatively charged moieties of the alginate/CNFs complex, i.e., the carboxylate groups, produce high zeta potentials, which stabilize the complexes in the aqueous electrolyte solutions.

Biocompatibility of Alginate/CNFs. Adsorbents of high biocompatibility are highly desirable in environmental remediation, especially in the case of in situ elimination of the targeted pollutants from the contaminated animals. In the in vitro experimental study with normal human fibroblasts (HF) as the typical cells, the relative cell growth (RCG), which was calculated by dividing a mean value of the treated cells by a mean value of untreated cells (cultured with 5% FBS), was found as high as $100 \pm 5\%$ at 1-day and 2-days after the administration of Na^+–ALG/CNFs and/or Na^+–ALG. RCG were better than $85 \pm 5\%$ even at 7-days after the administration. On the other hand, in the in vivo experimental study with rats as the typical animals, an increase in white blood cells (WBC) was observed at 1-week and 2-weeks after the administrations (WBC, μL^{-1}, increased from 5100 in the untreated rat to 7700 and 8100 in the rats given the control, while they increased to 7900 and 8400 for the rats given the Na^+–ALG/CNFs colloids). The β-globulin fraction also increased from 16.2% in the untreated rat to 21.7% and 21.5% in the rats treated with Na^+–ALG and/or Na^+–ALG/CNFs. Deep plica of the glandular stomach were observed at 1-week after the administration with Na^+–ALG and/or Na^+–ALG/CNFs. Changes in the other parameters were smaller than $\pm 10\%$. All hematological and biochemical data recovered to normal levels 3-weeks after the administration. Both in vitro- and in vivo-experiments showed that alginate and the alginate/CNFs complexes had no or very little detrimental impact on the cells/animals studied in this study.

Adsorptive Capability of the Vesicles. Three gelling solutions, (i) an aqueous solution containing sodium alginate alone at 20.0 mg/mL, (ii) CNFs at 0.5 mg/mL highly-dispersed in 20.0 mg/mL Na^+–ALG solution, and (iii) CNFs at 0.5 mg/mL simply dispersed (mixed by ultra-sonication for 5 min) in 20.0 mg/mL Na^+–ALG solution, were prepared and used for preparing the reference vesicles (those did not contain CNFs), the vesicles containing highly dispersed CNFs, and the vesicles containing low-dispersed CNFs, respectively. A typical microscopic observation of the vesicles containing highly dispersed CNFs are shown in Fig. 6. Vesicles having a diameter in the range of 400–800 micrometers were observed. The nozzle diameter strongly influenced the vesicle size. Generally, vesicles are produced with an average diameter two times larger than the nozzle diameter. However, the average vesicle diameter can be adjusted by about $\pm 20\%$ by varying the jet velocity and the vibration frequency. Kilogram quantities of the vesicles can be produced within a few hours using this encapsulation system. The Ba^{2+}–ALG/CNFs composite vesicles are both chemically and mechanically stable, and are much heavier than water (hence it is easy to isolate the vesicles from water).

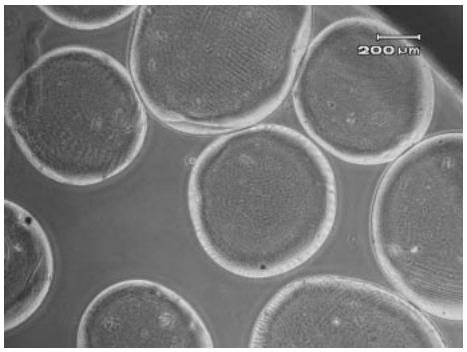

Fig. 6. Microscopic observation of the Ba^{2+}–alginate coated vesicles containing highly dispersed CNFs. The vesicles were produced using an aqueous colloidal solution containing 0.50 mg/mL CNFs and 20.0 mg/mL Na^+–ALG as the gelling solution with an aqueous solution containing 100 mM $BaCl_2$ as the gelatinizing solution.

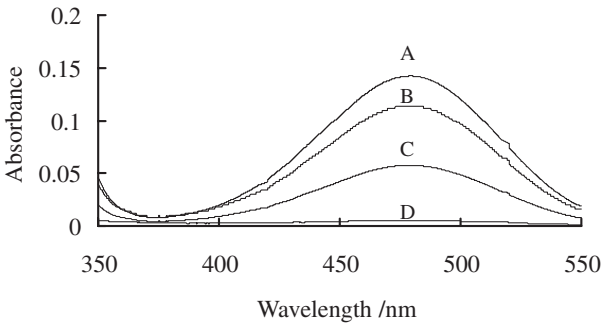

Fig. 7. UV–vis absorptions of an aqueous solution containing 30.0 μM ethidium bromide (A-trace); 15 mL of this 30 μM ethidium bromide solution being mixed with, the Ba^{2+}–alginate vesicles (reference vesicles; B-trace), the vesicles containing low-dispersed CNFs (C-trace), and the vesicles containing highly dispersed CNFs (D-trace) at 10 min after the solution/vesicles mixing.

Ethidium ions were chosen as the typical DNA-interactive species. They are highly water-soluble and used worldwide as an intercalating fluorescent dye for detecting DNA. Fifteen millilitres of the model contaminated water (prepared by dissolving ethidium bromide in deionized water at a concentration of 30.0 μM) was mixed with 10.0 mL of the vesicles. At about ten minutes after the mixing, approximately 3 mL of the bulk-phase solutions were collected and were measured using a UV–vis spectrometer. Figure 7 shows the results. The absorbance of the model contaminated water before the treatment, i.e., the 30.0 μM ethidium bromide aqueous solution, at

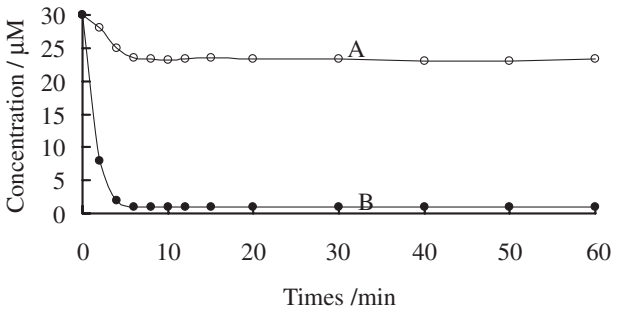

Fig. 8. Changes in concentrations of ethidium ions as a function of the contact time for a 15.0 mL of an aqueous solution containing 30.0 μM ethidium bromide after mixing that solution with 10.0 mL of the reference (Ba^{2+}–ALG) vesicles (upper-trace) and with the Ba^{2+}–ALG/CNFs composite vesicles (lower-trace).

480 nm was as high as 0.142. This, however, dropped to 4.9×10^{-3} after mixing this solution with the vesicles containing highly dispersed CNFs. Mixing the contaminated water with the vesicles containing low-dispersed CNFs also resulted in lowering the absorbance (which dropped to 0.058). This change, however, was much smaller than that observed for the vesicles containing highly dispersed CNFs. The control vesicles showed very little capability in trapping ethidium ions, suggesting the uptake of the targeted species is governed by the CNFs.

Figure 8 shows changes in the concentration of ethidium ions in the aqueous solution as a function of the contact time (15.0 mL of the 30.0 μM ethidium bromide aqueous solution were mixed with 10.0 mL of the vesicles containing the highly dispersed CNFs). The concentration of ethidium ions in the aqueous solution decreased rapidly as the contact time increased. It reached 0.98 μM and remained unchanged at about 8 min after mixing the solution with the Ba^{2+}–ALG/CNFs composite vesicles. In other words, 10.0 mL of the Ba^{2+}–ALG/CNFs composite vesicles took 0.44 μmol of the ethidium ions up and this uptake was accomplished within 8 min. The adsorptive experiments were performed three times, and the average value of the capacity for trapping ethidium ions for 10.0 mL of the vesicles obtained using the aqueous Na^{+}–ALG/CNFs colloidal solution containing 0.5 mg/mL for CNFs and 20.0 mg/mL for Na^{+}–ALG as the gelling solution was as high as 0.43 μmol (RDS $< \pm 1.2\%$).

Identical experiments were also performed using Ba^{2+}–alginate coated vesicles containing highly dispersed multi-walled carbon nanotubes (MWCNTs) as the adsorbents. MWCNTs (diameter, 20–40 nm; length, 1–5 μm, purity > 90%, as recommended by the manufacturer) were purchased from Nano Lab. and were used as received. Methods for preparing the aqueous Na^{+}–ALG/MWCNTs colloids, Ba^{2+}–ALG/MWCNTs composite vesicles, and for studying the capability for trapping ethidium ions were the same as for the CNFs. The capacity for trapping ethidium ions was found to be 0.42 μmol (RDS $< \pm 1.3\%$, $n = 3$) for 10.0 mL of the Ba^{2+}–ALG/MWCNTs vesicles obtained using an aqueous solution containing 0.50 mg/mL MWCNTs and 20.0 mg/mL Na^{+}–ALG as the gelling solution. This capacity value is virtually identical to that of the Ba^{2+}–ALG/CNFs composite vesicles, indicating the ethidium ions were unable to distribute in to the wall-nanopores of the CNFs. In other words, to accumulate the DNA-interactive chemicals in the wall-nanopores, it is necessary to expand the inter-planar distance among the graphite platelets of the CNFs.

Conclusion

Dispersing carbon nanofibers and/or nanotubes with a high uniformity into aqueous solutions is crucial for achieving the goal of utilizing these fascinating materials as high-performance adsorbents for absorbing/eliminating DNA-interactive type chemicals. The major goal in this study was achieved using alginate as the dispersing agent. Alginate is a non-toxic, naturally occurring linear polysaccharide (found widely in brown seaweed). Its gelling properties, together with its high biocompatibility, can generate a number of analytical-chemical, environmental, and biotechnological applications. Our dispersing approach based on alginate provides advantages over the previous methods:[19–25] (i) aqueous colloidal solutions containing CNFs and/or CNTs in pristine forms can be prepared in large-scales in a single step with a very simple manipulation procedure, (ii) the resultant aqueous colloids are highly biocompatible, and (iii) the colloids can be rearranged further as vesicles, providing a desirable approach to utilizing these nano-sized materials for macro-scaled applications. The selectivity of the Ba^{2+}–ALG/CNFs (also the Ba^{2+}–ALG/CNTs) composite vesicles for adsorbing DNA-interactive chemicals, especially for aromatic compounds having planar structures, is high. This is because the hexagonal arrays of the carbon atoms of the graphite sheets are the functional sites for trapping the targets. Note that columns packed with the entire CNFs and/or MWCNTs may also provide high trapping capabilities. They are, however, impractical for large-scale water treatment. This is because of the high backpressure that must be overcome to drive water through the packed column.

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