Impact of carbodiimide crosslinker used for magnetic carbon nanotube mediated GFP plasmid delivery

Yuzhi Hao1,2,3, Peng Xu2,4, Chuan He1, Xiaoyan Yang1, Min Huang1, James Xing5,6 and Jie Chen1,2,4

1 Department of Electrical and Computer Engineering, University of Alberta, Edmonton, AB, Canada
2 National Institute of Nanotechnology, Edmonton, AB, Canada
3 School of Chemical Engineering, Hebei University of Technology, Tianjin, People’s Republic of China
4 Department of Biomedical Engineering, University of Alberta, Edmonton, AB, Canada
5 IntelligentNano Incorporated, Edmonton, AB, Canada
6 Cross-cancer Institute, Edmonton, AB, Canada

Received 3 February 2011, in final form 29 April 2011
Published 8 June 2011
Online at stacks.iop.org/Nano/22/285103

Abstract

1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is commonly used as a crosslinker to help bind biomolecules, such as DNA plasmids, with nanostructures. However, EDC often remains, after a crosslink reaction, in the micro-aperture of the nanostructure, e.g., carbon nanotube. The remaining EDC shows positive green fluorescent signals and makes a nanostructure with a strong cytotoxicity which induces cell death. The toxicity of EDC was confirmed on a breast cancer cell line (MCF-7) and two leukemic cell lines (THP-1 and KG-1). The MCF-7 cells mainly underwent necrosis after treatment with EDC, which was verified by fluorescein isothiocyanate (FITC) annexin V staining, video microscopy and scanning electronic microscopy (SEM). If the EDC was not removed completely, the nanostructures with remaining EDC produced a green fluorescent background that could interfere with flow cytometry (FACS) measurement and result in false information about GFP plasmid delivery. Effective methods to remove residual EDC on macromolecules were also developed.

Online supplementary data available from stacks.iop.org/Nano/22/285103/mmedia
(Some figures in this article are in colour only in the electronic version)

1. Introduction

Gene delivery is an important research topic worldwide which has great potential in targeted gene therapy and tissue engineering. However, due to the protective nature of mammalian and plant cell membranes, biological molecules such as DNA cannot effectively enter cells by themselves. The drug and gene delivery systems enabled by nanotechnology aim to target selected cells or receptors in the body. The aim is that nanoparticles will carry therapeutic payloads or genetic content into diseased cells, minimizing the side effects as the nanoparticles will only become active upon reaching their ultimate destination. For instance, drug or gene delivery with magnetic carbon nanotubes in joints using magnetic forces can be used for guiding and targeting the drug or gene [1].

For nanostructure-based biodrug or gene delivery, the drug or gene must be bound to a nanostructure. Due to the specific characteristics of DNA and proteins, general chemical linking methods are not suitable. Carbodiimide is a common crosslinker which can chemically join two or more molecules through a covalent bond and is especially useful when used with proteins and other biomolecules [2–8]. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) is a well-known carbodiimide crosslinker because it is a zero-length crosslinker and does not become a part of the final crosslink product between molecules. As a result, this crosslinker is widely used in immobilization procedures, immunogen preparation and crosslinking between small molecules and nanostructures [9, 10].
Since the discovery of carbon nanotubes (CNTs) in 1991 [11], they have been widely used in building nanoelectronics and various biomedical applications. Magnetic carbon nanotubes (mCNTs) have been developed for drug and gene delivery due to their stiff nature [1]. These CNTs can forcefully enter cells by following external magnetic fields. The diameters of mCNTs can be as small as 1–2 nm, which means that they only induce minimal damage to target cells. Green fluorescence protein (GFP) plasmid bound on nanostructures is commonly used for evaluating the transfection efficiency of the gene delivery system by using flow cytometry (FACS) and confocal microscopy [1, 12, 13].

Carboimide is often used to covalently bind biomolecules, such as proteins and DNA, to mCNTs. Cai et al [1, 14] have demonstrated the use of EDC to bind GFP plasmid and poly-L-lysine (PLL) with mCNTs for gene delivery in various cells. Because there are many microscale apertures in CNTs, small molecules, such as EDC, can be captured in these micro-apertures and are not easily removed. CNTs in particular, have strong adsorption ability [15–18]. Peigney et al [19] reported that the maximum surface area of multi-walled CNTs is 1315 m² g⁻¹. When plasmids and CNTs are crosslinked, EDC is difficult to remove completely due to the plasmid characteristics. Therefore, if not thoroughly washed, a large amount of EDC will remain in the final product. Although it is a common belief that the residual EDC will cause cell damage, and has to be removed after binding GFP with carbon nanotubes for any further GFP transfection experiments, the biological impact of EDC is a controversial topic. So far, no research groups have qualitatively and quantitatively studied the impact of EDC for gene delivery.

In this paper, we have evaluated the biological impacts and cytotoxicity induced by EDC remaining in the carbon nanotube–GFP gene delivery system. The toxicity of EDC was confirmed on a breast cancer line, MCF-7, and two leukemic cell lines, THP-1 and KG-1. The nanostructures with remaining EDC produced a green fluorescent background which interfered with fluorescence detection to result in false results in GFP plasmid delivery. Methods for removing remaining EDC were also developed.

2. Materials and methods

2.1. Chemicals

The 1-ethyl-3-(3-dimethylaminopropyl) carboimide hydrochloride (EDC), hexamethyldisilazane and poly-lysine used in this study were obtained from Sigma-Aldrich. The RPMI 1640 medium, Iscove’s Modified Dulbecco’s Medium (IMDM), fetal bovine serum and penicillin/streptomycin used in this study were obtained from Sigma-Aldrich (No. 519318). The CNTs were refluxed with 0.5 M HNO₃ for 48 h. The magnetic CNTs (mCNTs) were obtained by a Nd–Fe–B permanent magnet [1].

2.2. Carbon nanotubes

The carbon nanotubes (CNTs) were purchased from Sigma-Aldrich. The CNTs were refluxed with 0.5 M HNO₃ for 48 h. The magnetic CNTs (mCNTs) were obtained by a Nd–Fe–B permanent magnet [1].

2.3. CNT binding with GFP plasmid

5 µg GFP plasmid, 1 mg treated mCNT and 10 mg EDC were mixed in 5 ml MiliQ water for 1 h at room temperature. This mixture was centrifuged to obtain precipitated mCNTs. They were resuspended in 1 ml serum-free culture medium (RPMI 1640) immediately [1].

2.4. Cells

MCF-7, a human breast adenocarcinoma cell line, and THP-1 and KG-1, two acute human leukemia cell lines, were purchased from the American Type Culture Collection (ATCC HTB22, Rockville, MD, USA) and used in this study.

2.5. Cell culture

The MCF-7 cells were maintained in 10 cm tissue culture dishes in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The THP-1 cells were maintained in 75 ml tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The KG-1 cells were cultured in 75 ml tissue culture flasks in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cells were cultured in an incubator at 37 °C and 5% CO₂. The medium was changed every two or three days.

2.6. Experimental procedure for GFP delivery

The MCF-7 cells were harvested using 0.25% trypsin solution. After washing and resuspension in RPMI 1640 growth medium at 2 × 10⁵ ml⁻¹, 1 ml of cell suspension was seeded in a 35 mm tissue culture dish. After one day’s incubation, the cell confluency was around 70%. The cell medium was renewed with (i) the medium containing GFP/CNT or (ii) 1 ml of RPMI 1640 medium without FBS and P/S with a final EDC concentration of 0.06–0.5 mg ml⁻¹. The culture dishes were placed on a Nd–Fe–B permanent magnet for 1 h, then incubated for 3 h with 5% CO₂ at 37 °C. The medium was refreshed again with growth medium and then incubated for 22 h.

2.6.1. THP-1 and KG-1 cells

400 µl of 1% poly-lysine solution was poured into 35 mm tissue culture dishes for 30 min and shaken evenly. The THP-1 cells and KG-1 cells were washed and resuspended in 1× phosphate buffer solution (PBS) at 6 × 10⁵ ml⁻¹. 1 ml of cell suspension was seeded into 35 mm tissue culture dishes after poly-lysine solution was removed. After 30 min incubation in an incubator, the PBS was aspirated with (i) the medium containing GFP/CNT or (ii) 1 ml of RPMI 1640 medium without FBS and P/S with a final EDC concentration of 0.06–0.5 mg ml⁻¹. The culture dishes were placed on a Nd–Fe–B permanent magnet for 1 h, then incubated for 3 h with 5% CO₂ at 37 °C. The medium was refreshed again with growth medium and then incubated for 22 h.
2.7. Morphological images
The MCF-7 cells were examined with a video microscope (Motic AE 31 optical microscope equipped with a Moticam 2300 camera).

2.8. Flow cytometry measurement
After incubating in an incubator for 22 h, the MCF-7 cells were harvested using trypsin from a 0.25% trypsin solution. The MCF-7, THP-1 and KG-1 cells were centrifuged at 1200 rpm for 10 min. The collected cells were resuspended in 400 μl PBS. The DNA transfection efficiency was evaluated with flow cytometry (BD FACSCalibur, Flowcytometer, Becton Dickinson) at an excitation wavelength of 488 nm.

2.9. SEM images
The MCF-7 cells were fixed in 2% glutaraldehyde in 4% PEA/cacodylate buffer, pH 7.2, for 2 h at room temperature. After rinsing in PBS twice at room temperature, the specimens were dehydrated in a graded ethanol series (50, 70, 90 and 100%) and in a graded mixture series of ethanol and hexamethydisilazane (HMDS) (75:25, 50:50 and 25:75). After dehydration, the specimens were kept overnight in 100% HMDS at room temperature. Finally, the specimens were coated with gold–palladium in a HUMMER 6.2 sputtering system. The specimens were examined under an XL 30 emission scanning electron microscope operated at 20 kV.

2.10. FITC annexin V apoptosis detection
MCF-7 cells were seeded in 35 mm tissue culture dishes at 2 × 10^5 cells/dish the day before detection. On the second day, medium was changed with new RPMI 1640 without FBS or P/S and EDC was added to a final concentration of 0.5 mg ml^{-1}. After incubation in an incubator at 37 ℃ and 5% CO₂ for a series of times (10 min, 30 min, 1 h, 2 h, 4 h and 24 h), cells were collected, washed, resuspended in 100 μl 1× staining buffer and labeled with FITC annexin V and PI for 15 min at room temperature. After that, 400 μl 1× staining buffer was added into each sample. Apoptosis analysis was carried out through flow cytometry (BD FACSCalibur, Flowcytometer, Becton Dickinson).

2.11. UV–vis spectra for checking EDC
The UV–vis spectra were determined by a Thermo Evolution 60 UV instrument.

3. Results and discussion
3.1. The green fluorescence signal from the remaining EDC during the crosslinking reaction
3.1.1. Transfection efficiencies of the MCF-7 cells. After the MCF-7 cells are incubated with mCNTs binding with GFP plasmid through EDC, the FACS results show that the transfection efficiency is about 20%. It seems that the GFP plasmid is expressed in MCF-7 cells. However, in the same incubation conditions, the FACS results also show that the transfection efficiencies of EDC, the GFP/EDC mixture and the CNT/EDC mixture are above 20% when the EDC concentration is 0.15 mg ml^{-1} in figure 1. The FACS results indicate that the transfection efficiencies of the GFP plasmid, CNTs and GFP/CNT mixture are almost zero. However, as it is impossible that GFP can exist in MCF-7 cells incubated with EDC and CNT/EDC mixture, we can conclude that the transfection efficiencies in FACS result for MCF-7 cells incubated with EDC and CNT/EDC mixture are not from GFP plasmid. Because the transfection efficiency of MCF-7 cells incubated with EDC only is above 20%, it is reasonable to conclude that this transfection efficiency is from the EDC effect, not from the GFP plasmid.

3.1.2. The green fluorescence signals of MCF-7, THP-1 and KG-1 cells incubated with EDC. When pure EDC is incubated with MCF-7 cells, THP-1 cells and KG-1 cells, strong green fluorescence signals appear in FACS determination (refer to figure 2(A)). The green fluorescence signals detected are about 78% for MCF-7 cells, 84% for THP-1 cells and 88% for KG-1 cells after incubation with 0.5 mg ml^{-1} EDC, respectively. The morphology of the MCF-7 cells greatly changes after contact with EDC. There is more cell debris outside the selected region compared with the cell only, which displays more cell death for THP-1 and KG-1 cells. The results indicate that EDC cannot only produce green fluorescence signals in FACS, but also induce more cell death.

3.1.3. The green fluorescence signals of different EDC concentrations. Different EDC concentrations in the culture medium of cells can produce green fluorescence signals with different intensities (figure 2(B)). When the EDC concentration was between 0.06 and 0.5 mg ml^{-1}, the green fluorescence signal was 3–78% for MCF-7 cells, 52–84% for THP-1 cells and 13–88% for KG-1 cells. At lower EDC concentrations, THP-1 cells are more sensitive than the other two cell lines, and the green fluorescence signal is the strongest. For example, when the EDC concentration is 0.06 mg ml^{-1}, the green fluorescence signal is about 52% for THP-1 cells, 13% for KG-1 cells and about 3% for MCF-7 cells. The higher the EDC concentration is, the stronger the green fluorescence signal is. Therefore, when the EDC concentration is lower than 0.05 mg ml^{-1}, the green fluorescence signal for MCF-7 cells can be ignored.
3.1.4. The UV–vis spectra of EDC and filtrate of carbon nanotubes (CNTs) and EDC mixture. According to figure 3, the UV–vis absorbance at 225 nm from the filtrate of CNTs mixing with the same amount of EDC as the EDC only is obviously lower than that of the EDC only. This shows that EDC molecules in solution can be adsorbed onto CNTs. Because there is a large surface area and there are micro-apertures in CNTs, EDC can be adsorbed on the surface or into the apertures of CNTs. If these CNTs are not completely washed, these EDC molecules will accompany the CNTs into cells when the CNTs are used as vectors for gene delivery.

3.2. Hypothesis of EDC inducing cytotoxicity

3.2.1. EDC induced cell membrane damage. Protein is composed of peptides which consist of amino acids [20]. Free functional groups exist in many amino acids after peptide construction, such as hydroxyl (–OH) from serine, threonine and tyrosine; terminal amino (–NH$_2$ or –NH) from arginine, histidine, lysine, asparagine, glutamine and tryptophan; thiol (–SH) from cysteine; terminal carboxylic (–COOH) from aspartic acid and glutamic acid. These functional groups interact with each other to form hydrogen bonds or disulfide bonds which constitute the secondary structure of the protein. These functional groups have good nucleophilic ability, the order of their nucleophilic ability is $–\text{SH} > –\text{OH} > –\text{NH}_2 > –\text{OH}$ (in COOH).

The structure of EDC contains two C=O double bonds connected with the same carbon atom, thereby its reactivity is high. Due to the higher electronegativity of nitrogen, the carbon atom reacts easily with nucleophilic agents, which results in a nucleophilic addition reaction. EDC usually occurs as its HCl salt in the reaction. Because EDC is a line molecule and soluble, it can easily diffuse into proteins on a cell surface and react with the free functional groups in proteins, for example $–\text{SH}$, $–\text{OH}$, $–\text{NH}_2$, $–\text{OH}$ (in COOH) (scheme 1). The S, O, N atoms contain lone pairs of electrons, and they tend to react with atoms which have positive charge, such as the carbon atom in N=C=N. As a result, the reaction makes a C=N double bond open and forms a new C–O bond between EDC and –COOH. In this new structure, due to the strong inductive action from N=C–N, the C=O bond is activated. Most of the positive charge will be surrounding the carbon atom in C=O. Therefore, the C=O group in this structure will easily react with nucleophilic agents. Amino groups in a molecule, such as RNH$_2$, will react with the carbon atom in C=O and an addition–elimination reaction will take place, while a new amide bond is formed and a urea by-product is produced. Based on a similar principle, sulfur atoms in –SH or –S–S– groups and oxygen atoms in –OH groups have a stronger nucleophilic ability than nitrogen in –NH$_2$ groups. They will powerfully react with the carbon atom in N=C=N, and form
thiol urea or urea. The reaction products urea or thiol urea are also protein denaturing agents \[21, 22\].

After a nucleophilic addition reaction, the nucleophilic ability of S, O and N atoms in proteins decreases. Therefore, the ability of these atoms to combine with hydrogen also decreases. As a direct result, the hydrogen bonds between free functional groups of peptides are broken, and the secondary structures of proteins are broken as well. Therefore the tertiary structure and quaternary structure of proteins will be damaged. These damaged proteins will lose their biological functions.

An integral membrane protein is a protein which lies across the cell membrane \[20\]. Therefore, if integral membrane proteins are damaged, the cell membrane structure will be broken and thus there will be some holes left in the cell membrane. Cells will die after their membranes are broken. This result is verified by the SEM images from figure 4. For normal MCF-7 cells (figures 4(a) and (b)), there is a lot of proteins on the surface of the cell membrane. However, the amount of protein on the surface of the cell membrane obviously drops after incubation for 10 min in medium with 0.5 mg ml\(^{-1}\) EDC (figures 4(c) and (d)). The holes appear after MCF-7 cells are incubated with EDC.

Compared with normal MCF-7 cells (figure 5, left), the morphology of a few cells is changed after incubation in medium with 0.5 mg ml\(^{-1}\) EDC for 10 min (figure 5, left (b), black arrow). There are some pimple-like structures on these cell surfaces. After incubation for 20 min under the same conditions (figure 5, left (c)), the cell shape has changed for most cells. A lot of vesicle-like structures are produced on the surface of the cell membrane. After incubation for 45 min under the same conditions (figure 5, left (d)), there is severe damage on most of the cell surfaces while others stay the same as those incubated for 20 min. Compared to the cells in the control group, these cells become flatter after incubation with EDC. However, after incubation for 210 min under the same conditions (figure 5, left (e)), almost all of the cell’s morphologies are changed.

Figure 5, right presents the SEM images for the MCF-7 cells compared with figure 5, left. For normal MCF-7 cells (figure 5, right (a)), there is a lot of tomenta on the cell surface. The cells are plump. Some cells become flat after incubation in medium with 0.5 mg ml\(^{-1}\) EDC for 10 min (figure 5, right (b)). A few cells produce some pimple-like structures. After incubation for 20 min (figure 5, right (c)) under the same conditions, most of the cells produce a lot of pimple-like structures. After incubation for 45 min (figure 5, right (d)) under the same conditions, there is a big change in cell shape and the pimple-like structures became larger. Some of the pimple-like structures look like flowers. After incubation for 210 min (figure 5, right (e)) under the same conditions almost all of the cells changed shape. Most of the cells became flatter. Almost no tomenta existed on the cell surfaces. Bigger holes appeared on the cell membrane through which the internal cytoplasm came out. Some of the cytoplasm that came out looked like flowers (figures 5, right (d) and (e)). All these images show that MCF-7 cell membranes are damaged significantly after contact with EDC. The MCF-7 cells die due to their damaged membranes.

3.2.2. The cytotoxicity of EDC and the cell death process.

There are two main processes for cell death, necrosis and apoptosis. Loss of plasma membrane is one of the earliest features in apoptosis of cells. The membrane phospholipid-phosphatidylyserine (PS) is translocated from the inner to the outer section of the plasma membrane, exposing it to the external cellular environment. Annexin V is a 35–36 kDa Ca\(^{2+}\) dependent phospholipid-binding protein which has a high affinity for PS and can bind with exposed PS. FITC annexin V staining precedes the loss of membrane integrity.
Figure 4. SEM images of MCF-7 cells before and after incubation for 10 min with 0.5 mg ml\(^{-1}\) EDC. (a) Individual normal MCF-7 cells; (b) zoomed-in image of the area circled in image (a); (c) MCF-7 after incubation for 10 min with 0.5 mg ml\(^{-1}\) EDC; (d) zoomed-in image of the area circled in image (c). The white extravasation is the cytoplasma coming out from the cell.

Figure 5. Microscope and SEM images of MCF-7 cells after incubation for different times with 0.5 mg ml\(^{-1}\) EDC. (a) Before incubation; (b) incubated for 10 min; (c) incubated for 20 min; (d) incubated for 45 min; (e) incubated for 210 min.
Figure 6. FACS images of FITC annexin V staining for MCF-7 cells after contact with 0.5 mg ml\(^{-1}\) EDC for different amounts of time. which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC annexin V is typically used in conjunction with a vital dye, such as propidium iodide (PI), to identify early apoptotic cells (PI negative, FITC annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Therefore, cells that are considered viable are FITC annexin V and PI negative; cells that are in early apoptosis are FITC annexin V positive and PI negative; cells that are in late apoptosis or already dead are both FITC annexin V and PI positive.

Figure 6 shows the FACS images of FITC annexin V staining MCF-7 cells after contact with EDC for different amounts of time. After contact with 0.5 mg ml\(^{-1}\) EDC for 10 min, 30 min, 1 h, 1.5 h, 2 h and 4 h, the cell number that appears in the lower right region is at the same level as that of the control group. This shows that FITC annexin V is negative for MCF-7 cells in contact with EDC for less than 4 h. However, the cell number becomes more and more concentrated in the upper left region from 0.76 to 63.19. This shows that PI is positive for MCF-7 cells in contact with EDC for less than 4 h. Almost all MCF-7 cells are dead in the upper right region after contact with 0.5 mg ml\(^{-1}\) EDC for 24 h. All of these results show that MCF-7 cell death at the earliest stage precedes necrosis, not apoptosis. The supplemental information (available at stacks.iop.org/Nano/22/285103/mmedia) confirms this conclusion.

Based on our previous observations, we suggest that the process for MCF-7 cell death is: (1) the cell shapes become flat and some holes appear on the cell membrane; (2) the internal cytoplasm comes out through these holes, forming pimple-like sections; (3) more and more internal cytoplasm come out as the holes grow bigger and bigger; (4) the cells die.

3.3. Hypothesis of EDC producing green fluorescence

Transport proteins are a kind of integral proteins. Therefore, if a transport protein is damaged, the cell membrane structure will be damaged. In fact, there are holes left in the cell membrane (figure 4(d)) after the cell surface proteins are damaged. After the cell membrane is damaged, EDC can easily go into the cell and damage other proteins inside. For example, EDC can also destroy the phosphatases which
exist commonly in cells. The phosphatases contain the Ser–Tyr peptide sequence [23, 24] and the Thr–Tyr peptide sequence [25, 26]. These two peptide sequences form similar peptide structures. Therefore, Ser–Tyr–any amino acid (or Thr–Tyr–any amino acid) structures can be easily formed (scheme 2) after proteins are denatured in cells. This structure can easily combine with free –COOH and –OH groups from other peptide chains inside the cell to form a conjugation structure which is similar to the GFP fluorescence structure [27], which can produce green fluorescence in FACS determination.

Although the cell membranes are damaged by EDC, these cells do not become debris (figure 7) after an extra 22 h of incubation in renewing medium and may appear in the selected region in FACS determination (figure 2, left). Inside the damaged cells it is possible that similar GFP fluorescence structures are formed, which display a false transfection efficiency in FACS determination. This is the main reason why cells can produce an FACS transfection efficiency background after contact with EDC.

### 3.4. Methods of reducing the green fluorescence background of EDC

#### 3.4.1. Effect of using NHS accompanied with EDC on the green fluorescence background

When EDC binds –COOH groups in CNTs or polymer with –NH2 groups in biomolecules, or vice versa, adding NHS (N-hydroxysuccinimide) can increase the product yield. According to figure 8, using NHS can also decrease the fluorescence background from about 37% to 5% under the same conditions. It is possible that NHS can quickly react with the active O-acylsourc intermediate produced by EDC, which makes the probability of a reaction occurring between the EDC and proteins decrease. Therefore, the green fluorescence background is reduced by NHS.

#### 3.4.2. Effect of the ethanol precipitated DNA method on removing remaining EDC

When EDC binds CNTs or polymer with DNA, the DNA can be precipitated in ethanol. The CNTs or polymer will precipitate together with DNA, but the EDC will remain in ethanol solution. By removing the supernatant, extra EDC will also be removed. Figure 9(A) shows the reaction solution of EDC, CNTs and GFP plasmid after washing twice by ethanol, almost no EDC remained in the final DNA solution. Figure 9(B) further confirms that no...
green fluorescence background exists after ethanol washing of the reaction solution of EDC, CNTs and GFP plasmid twice.

4. Conclusion

The remaining EDC during the binding reaction can induce cell death and produce an FACS background in GFP plasmid transfection. The reason for the cell death is that the EDC can react with the functional groups in the proteins of the cell surface and destroy the secondary structure of the proteins, which damages the cell membrane. The cell death comes from necrosis, not apoptosis. The peptide sequences from the damaged proteins inside the cells may combine with each other to form GFP fluorescence structure, which produces green fluorescence background in FACS determination. The EDC induced green fluorescent signal can affect the FACS results and it is important to completely remove the EDC before performing gene delivery experiments. The use of NHS accompanying the EDC or the ethanol precipitated DNA method can reduce this FACS background.

Acknowledgments

The authors would like to acknowledge the funding support of an NSERC and CIHR joint CHRP grant, and an NSERC engagement grant. We would like to thank Hilal Gul for her valuable input and Scott MacKay for proofreading the manuscript.

References

[24] FermiMn E and Domínguez A 1997 Microbiology 143 2615

Figure 9. (A) UV–vis spectra of remaining EDC in the supernatant by using the ethanol precipitated DNA method. (B) FACS image of the decreasing effect of green fluorescence background by using the ethanol precipitated DNA method.