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Development of amine-containing polymeric particles

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Abstract—The objective of this study was to synthesize and characterize particles as a drug-delivery platform for gliomas, a highly advanced and invasive stage of brain tumor with poor prognosis. Poly(aminoethyl methacrylate-co-methyl methacrylate) particles were prepared by suspension polymerization and poly(aminoethyl methacrylate-co-poly(ethylene glycol) methacrylate) particles were prepared by emulsion (w/o) polymerization. Amine groups of the particles were complexed with tetrachloroplatinate to form a cisplatin-like molecule. Particles were characterized with respect to size, ζ-potential, amine content, loading efficiency and drug release. Poly(aminoethyl methacrylate-co-methyl methacrylate) particles had diameters of below 10 μm, whereas the poly(aminoethyl methacrylate-co-poly(ethylene glycol) methacrylate) particles had diameters of approx. 1 μm. Poly(aminoethyl methacrylate-co-poly(ethylene glycol) methacrylate) particles had a more positive ζ-potential as compared to poly(aminoethyl methacrylate-co-methyl methacrylate) particles, although the amino-group content of both particles was almost equivalent. The net positive charge on the particles decreased after complexation with tetrachloroplatinate for both types of particles. Both particles had very high platinum-loading efficiency (>85%) and showed slow release of platinum over time. Particles had relatively low cytotoxicity (LC_{50} > 100 μg/ml) and demonstrated a high degree of association with cells. Complexation with poly(aminoethyl methacrylate-co-methyl methacrylate) particles significantly reduced the toxicity of platinum. The poly(aminoethyl methacrylate-co-poly(ethylene glycol) methacrylate) particles have potential for being an effective drug-delivery platform and continued investigation is warranted.

Key words: Polymer; particles; aminoethyl methacrylate; drug delivery.

INTRODUCTION

Polymeric systems are being widely explored for delivery of small molecules and proteins, gene therapy and tissue engineering. These systems include soluble macromolecular polymer conjugates, particles and insoluble matrices. Particles

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have shown great potential for drug delivery. Drug molecules may be encapsulated in, coated on, or incorporated into the backbone of particles [1]. Most particulate systems described to date are formed by encapsulating drug inside the particles or adsorbing drug to particles [2, 3]. Few studies have been reported using complexation mechanisms to control the release of a drug from a polymeric particle. In this report we have studied amine containing particles for delivery of anti-cancer agents, specifically platinum compounds, to brain tumors.

Gliomas are highly invasive brain tumors with poor prognosis. The delivery of chemotherapeutic agents to the brain is hindered by the blood–brain barrier. Also, systemic administration of these agents leads to toxicity. These major problems can be resolved by direct intracerebral injection of drug-loaded particles. Particles can be sterotactically injected into the tumor site in the brain by creating a small hole in the skull. This technique is less invasive than surgical implants that are considered the primary treatment for many brain tumors. Local administration can, thus, be more effective and reduce systemic toxicity. Various chemotherapeutic agents, including 5-fluorouracil, carboplatin and cisplatin, encapsulated in poly(D,L-lactide-co-glycolide) particles, have been found to be more effective after intracranial implantation [4–6]. Although particulate drug-delivery systems have gained increasing acceptance in recent years [7], several problems have typically been associated with particulate drug-delivery systems, including recognition by the immune system. Studies have shown that poly(ethylene glycol) (PEG) reduces the immunogenicity and protein antigenicity by reducing immune system recognition [8]. Poly(ethylene glycol) diacrylate (PEGDA) hydrogel membranes have shown potential for xenoprotection after implanting foreign cells [9]. Thus, the use of PEGDA in hydrogel particles may reduce recognition by the immune system and suggest the use of PEGDA as a component of hydrogel particles for cancer chemotherapeutic delivery for brain tumors.

Hydrogels and hydrated hydrogel particles have not been extensively utilized for small molecule drug delivery due to rapid release of entrapped drug. Drug release from hydrated polymers and hydrogels is quite rapid and determined by diffusional release; recently, some systems have been developed that retard diffusion using molecular interactions including ionic interactions, conjugation and complexation [10, 11]. To treat cancer, particularly in the brain, we have proposed to exploit platinum complexation with amines to control release of the drug from polymeric particles directly injected into the brain.

This paper explores two techniques for particle preparation, namely suspension polymerization [12] and emulsion (w/o) polymerization [13]. We have developed and compared two amine containing cross-linked particulate systems with respect to their physical properties and cytotoxicity. Aminoethyl methacrylate (AEMA, Fig. 1), is an amine-containing monomer used in both of these particles. A co-monomer was required since it was observed that AEMA alone does not form particles using the methods described here (preliminary data not shown). One system used methyl methacrylate (MMA) as a co-monomer to make the particles,

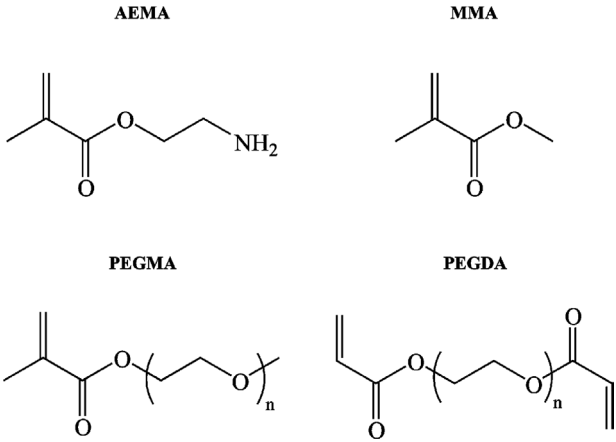

Figure 1. Schematic representation of the chemical structures of monomers used in this study: aminoethyl methacrylate (AEMA), methyl methacrylate (MMA), poly(ethylene glycol) methacrylate (PEGMA) and poly(ethylene glycol) diacrylate (PEGDA).

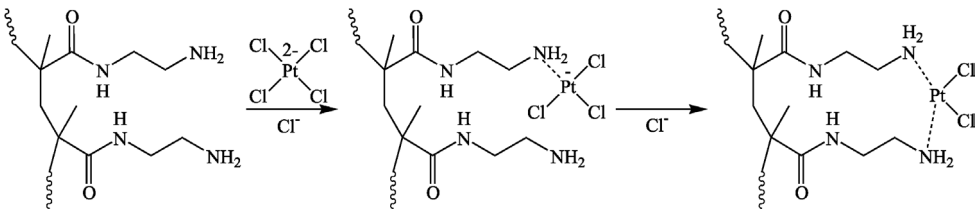

Figure 2. Schematic representation of the chemistry of interaction between AEMA and tetrachloroplatinate. Potassium tetrachloroplatinate is added to the particle suspension which reacts with the amino groups of AEMA releasing a chlorine atom. A second reaction with an amine from AEMA is also possible. The second amine group may be neighboring, local on that chain, local on that particle, or on another particle.

while the other utilized poly(ethylene glycol) methacrylate (PEGMA). The amino groups on the particles were complexed with tetrachloroplatinate to form a molecule similar in structure to the second-generation cisplatin derivative, iproplatin (Fig. 2) [14]. Platinates have been shown to be effective in the treatment of many forms of cancer, including malignant gliomas, particularly when administered locally [15–17].

Particles were characterized with respect to particle diameter, ζ-potential, amine content and drug release. Particle cytotoxicity was tested using U-87MG cells as a model. Further, we studied the interaction of particles with cells by epifluorescent microscopy. These particles have been designed to meet the important needs of an ideal delivery system and resolve the main problems of delivery, which may eventually lead to successful treatment strategies. Since they may have the advantages of long circulation with reduced systemic toxicity and passive targeting to tumor cells, they may have a great potential as a delivery system for many types

of therapeutic agents. If successful, these particles may be applicable to a wide range of delivery options and to determine properties to be used for designing the next generation of particles.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA) and used without purification unless otherwise described.

Preparation of particles

AEMA–MMA microparticles were prepared by suspension polymerization (Fig. 3A), using ammonium persulfate as an initiator similar to that previously described [18]. Poly(ethylene glycol) diacrylate (PEGDA) was included as a cross-linker at a final solution concentration of 2 mM. AEMA was fed into the monomer mixture as mol fractions (X_{AEMA}) of 0.44 with the total monomer concentration of 40 mM. The reaction mixture was stirred at 1000 rpm for 5 h at 70°C and particles were separated after cooling to room temperature by centrifugation for 15 min at 2×10^4 rpm.

AEMA–PEGMA microparticles were prepared by emulsion polymerization (Fig. 3B) [19]. The cross-linker (PEGDA, 8.4 mM) and initiator were the same

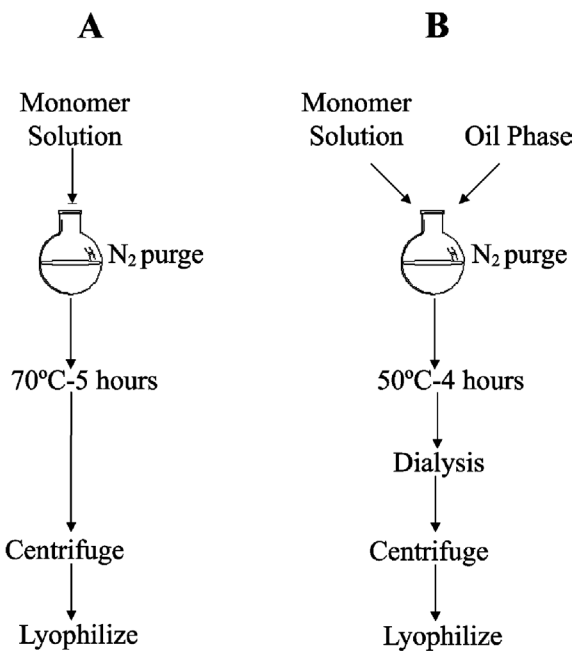

Figure 3. Schematic description of the preparation methods and for (A) polymerization of AEMA–MMA nanoparticles and (B) polymerization of AEMA–PEGMA nanoparticles.

as for AEMA–MMA particles. AEMA was included in monomer mixtures at a mol fraction of 0.38 with a monomer concentration of 510 mM in the water phase. Monomers, cross-linker and initiator were dissolved in water and this solution was added to the organic phase (hexanes) containing Span 80 (0.7 mM) and Tween-80 (0.2 mM) (Fisher Scientific, Pittsburgh, PA, USA) as surfactants. The emulsion was stirred for 5 h at 50°C. Hexanes were evaporated and the surfactant residue was dialyzed against acetone, hexane/acetone (1 : 1) and acetone/water (1 : 1) to remove the surfactants. Particles were separated by centrifuging for 20 min at 1×10^4 rpm, washed, centrifuged and lyophilized.

Complexation of platinum

Particles were sonicated for 30 min and a solution of potassium tetrachloroplatinate in water was added at a molar ratio of 2 : 1 (amine/drug) and stirred overnight. Particles were separated by centrifuging for 20 min at 1×10^4 rpm, washed, centrifuged and lyophilized. Drug loading was determined by analyzing the drug concentration in the supernatant solution using *o*-phenylene diamine (OPDA) [20].

Particle analysis

Particle diameter and size distribution was determined using dynamic light scattering (NICOMP 370 particle sizer). Particles were suspended in phosphate-buffered saline (PBS) and sonicated for 30 min before analysis. ζ-potential was measured (Model 501 Lazer Zee Meter; Pen Kem, Bedford Hills, NY, USA) and compared to standard ζ-potential controls (Laszlo Kovacs, LLC).

Amine content was determined using the colorimetric reaction of ninhydrin with primary amines [21]. Briefly, blank particle suspension (1 ml) was added to 500 μl ninhydrin reagent (1%, w/v) and heated in a boiling water bath for 15 min. The total volume was made up to 5 ml and cooled to room temperature, and UV absorbance was measured at a wavelength of 564 nm. AEMA was used to prepare the calibration curve.

Platinum release

A suspension of 1 mg/ml (5 ml) of platinum-complexed particles was placed in a dialysis bag, which was placed in PBS. The release of Pt from the particles into PBS was determined at various time intervals using OPDA.

Cell culture

The U-87MG (ATTC #HTB-14) cell line was grown and maintained in Eagle Minimum Essential Media with 10% fetal bovine serum, 1% penicillin/streptomycin, sodium pyruvate (110 mg/l), L-glutamine (292 mg/l) and non-essential amino acids. Cells were cultured at 37°C in 5% CO_2.

Table 1.
Particle diameter determined using photon correlation spectroscopy

Particle	Diameter in nm (% fitting that population)		
	Population 1	Population 2	Population 3
Unloaded			
AEMA–MMA	518 ± 100 (2.5)	6365 ± 1282 (97.5)	
AEMA–PEGMA	97 ± 15 (51)	476 ± 101 (49)	
Pt-loaded			
AEMA–MMA	28 ± 4 (21.1)	1079 ± 167 (10.9)	9056 ± 1050 (68)
AEMA–PEGMA	64 ± 14 (33.9)	1052 ± 222 (66.1)	

All size fractions are presented as mean ± standard deviation with percent of particles fitting that population in parentheses.

Cells were plated at 5×10^4 cells/ml in 96-well plates and incubated for 24 h. Varying concentrations of the loaded or unloaded particles were incubated with the U-87MG cells for 24 h. Cell viability with respect to the untreated control was calculated using a modified MTT [22] assay (Cell Titer 96® Aqueous One solution Cell Proliferation Assay; Promega, Madison, WI, USA). UV absorbance at a wavelength of 492 nm was measured and compared to the untreated control.

Particle–cell interaction

To determine the association between particles and cells, unloaded particles were labeled with Alexa Fluor® succinimide 350 as described by the manufacturer (Molecular Probes, Eugene, OR, USA). Labeled particles were incubated for 24 h with U-87MG cells. Cells were stained with ethidium homodimer and calcein (Live/Dead® viability/cytotoxicity kit; Molecular Probes) and observed under an Olympus IX70 inverted epifluorescence microscope with appropriate filters (Chroma Technologies, Rockingham, VT, USA). Images were captured and analyzed using IPLab software (Scanalytics, Fairfax, VA, USA).

RESULTS

Production of particles

AEMA–MMA particles were found to have a multimodal distribution in PBS (Table 1). AEMA–PEGMA particles also have multimodal distributions in PBS with nearly equal fractions. Platinum-complexed particles show larger diameters compared to the unloaded particles in both formulations due to bridging or cross-linking by tetrachloroplatinate. However, loaded AEMA–PEGMA particles show less cross-linking of particles and, hence, smaller diameters as compared to AEMA–MMA particles (Table 1).

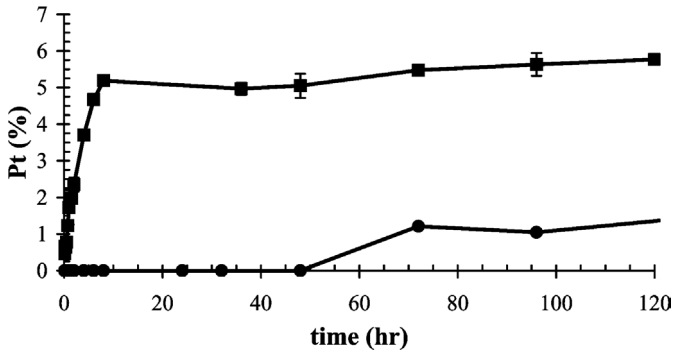

Figure 4. Cumulative release of platinum from AEMA–MMA particles (■) and AEMA–PEGMA particles (●).

The ζ-potential of AEMA–MMA particles was $+14 \pm 1$ mV and that of AEMA–PEGMA particles was $+25 \pm 3$ mV. The ζ-potential of the particles decreased after complexation with platinum. The platinum-complexed AEMA–MMA particles had a ζ-potential of -3.1 ± 1.2 mV, whereas AEMA–PEGMA particles had a ζ-potential of $+15.9 \pm 1.2$ mV. AEMA–MMA particles have 1.84 ± 0.08 mM of amino groups and AEMA–PEGMA particles have 1.86 ± 0.06 mM amino groups/g dry weight of particles.

AEMA–MMA particles had a loading efficiency of $87.9 \pm 0.6\%$, whereas AEMA–PEGMA particles had a loading efficiency of $96.37 \pm 0.25\%$. Both particle types demonstrate a high capacity to load the drug when loaded from a solution containing one platinum atom for every two amine groups in the particle.

AEMA–MMA particles showed an initial burst release of the drug in the first 8 h and then reached a plateau which steadily increases over time (Fig. 4). AEMA–PEGMA particles did not show a burst release profile (Fig. 4), but did show a maintained release over the course of delivery. Platinum release of the drug from both particles is sustained over time.

Particle–cell interaction

AEMA–PEGMA particles were less toxic to cells than AEMA–MMA particles. The LC_{50} for AEMA–MMA particles was found to be 259.2 μg/ml and that for AEMA–PEGMA particles was 951.99 μg/ml. The platinum-complexed AEMA–MMA particles show an LC_{50} of 931.29 μg/ml, whereas the platinum-complexed AEMA–PEGMA particles have an LC_{50} of 325.28 μg/ml. In comparison, the LC_{50} of cisplatin is 37.47 μM and of potassium tetrachloroplatinate is 69.53 μM. The decrease in short-term platinum toxicity from 40–70 μM (cisplatin or tetrachloroplatinate) to 1427 μM (Pt equivalent in AEMA–MMA particles) or 155.5 μM (Pt equivalent in AEMA–PEGMA particles) was significant.

Unloaded particles are associated with cells as seen in the cell interaction studies (Figs 5 and 6). The positive surface charge on the particles may enhance their

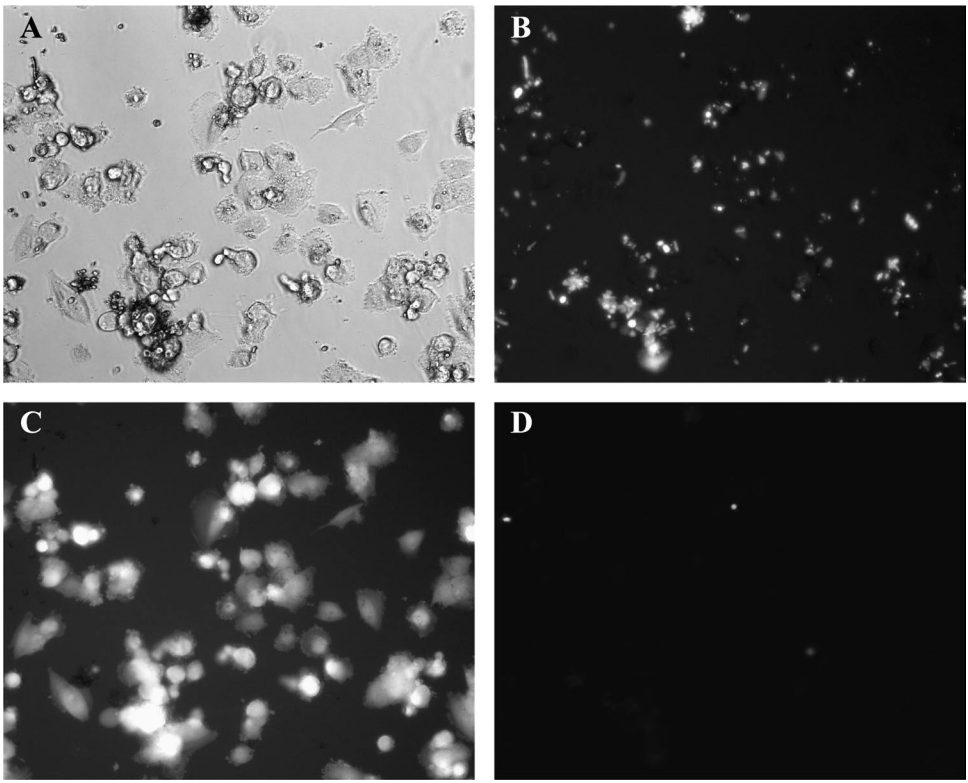

Figure 5. Malignant glioma cells treated with 50 μg/ml AEMA–MMA nanoparticles. (A) Cells viewed with phase contrast. (B) The nanoparticles as viewed by fluorescent imaging of Alexa Fluor® 350 labeled images. (C) Live cells were stained with calcein and (D) dead cells were stained with ethidium homodimer.

interaction with the negatively-charged cell membrane. Almost all of the cells, including those associated with particles, were alive as indicated by the large bright areas (Figs 5C and 6C) indicative of calcein accumulation. In all cases there are very few dead cells (Figs 5D and 6D) as determined by ethidium homodimer accumulation. There appears to be minimum toxicity arising, even after the association of the particles with the cells. These results support those obtained from the toxicity assay, indicating that the particles are only mildly toxic to the cell line examined.

DISCUSSION

This study explored and characterized a potential drug-delivery system. We have been able to produce two types of hydrogel particles of appropriate size for administration via various routes. Each of the two formulations has properties that would be advantageous for *in vivo* use, although the PEGMA-containing particles

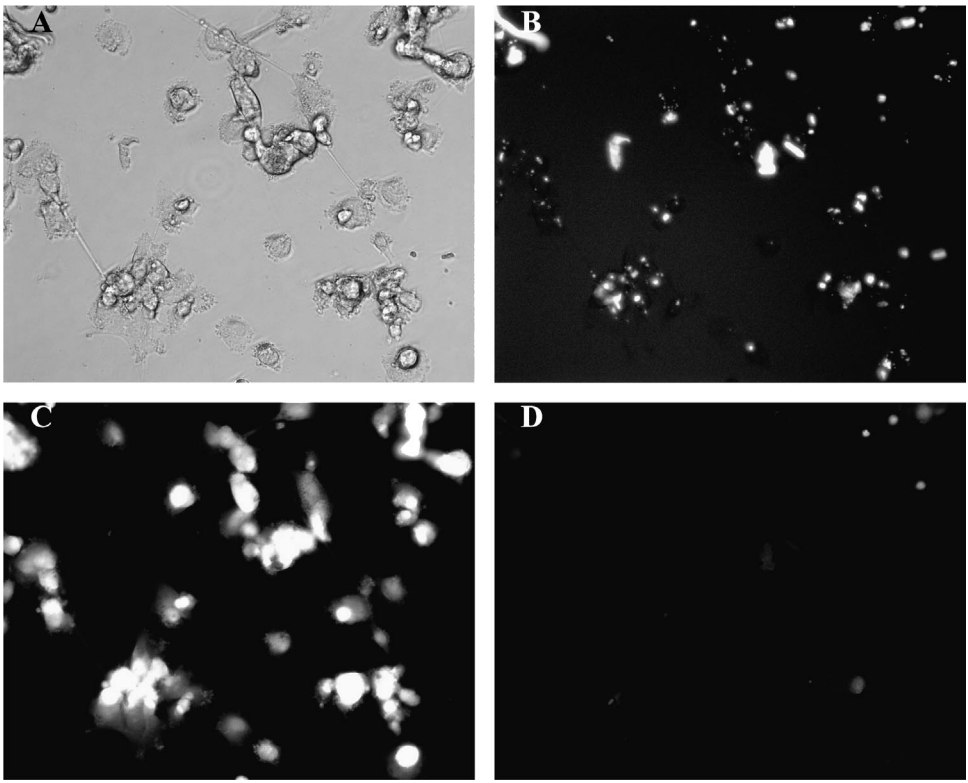

Figure 6. Malignant glioma cells treated with 50 μg/ml AEMA–PEGMA nanoparticles. (A) Cells viewed with phase contrast. (B) The nanoparticles as viewed by fluorescent imaging of Alexa Fluor® 350-labeled images. (C) Live cells were stained with calcein and (D) dead cells were stained with ethidium homodimer.

are expected to have a limited cellular and immune recognition. Synthesized particles may be especially useful for intracerebral administration, since they are unable to escape the blood–brain barrier and will be retained at the tumor site [23]. Further studies are necessary to confirm the toxicity, distribution and retention time of these particles before they can be suggested to be applied in the clinic. However, even if the designed particles are not clinically applicable, these particles will be used to better understand the properties necessary to design the next generation of particulate drug delivery.

The ζ-potential of AEMA–MMA particles is less than that of AEMA–PEGMA particles, although the number of amino groups present per gram of particles is almost equivalent for both types of particle. This may be due to the distribution of the amino groups within the particles during polymerization. MMA, being more hydrophobic, may be oriented towards the outer surface of the particles in the w/o emulsion, creating an environment where AEMA–MMA particles may have more amino groups masked in the interior of the particles due to a hydrophobic

MMA shell [24], whereas AEMA may be evenly distributed in AEMA–PEGMA particles. The ζ-potential difference is important because the increased surface charge may prevent aggregation of the particles [25, 26]. The stability of AEMA–PEGMA should be additionally augmented by the pendant PEG chains present on the particle surface. The charge and number of amino groups present can be manipulated by varying the molar ratio of the co-monomers. Optimization of the required charge and functional groups is possible; however, slight variations in co-monomer composition greatly affect the particle diameter and distribution obtained (data not shown).

Particles alone show limited toxicity at low concentration; toxicity begins as turbid solutions of the polymeric particles are formed. Drug toxicity has also been considerably reduced due to complexation. The low toxicity of the platinum-complexed AEMA–MMA particles compared to unloaded AEMA–MMA particles can be explained by the differences in their diameters and charges. Platinum-complexed AEMA–MMA particles have a significantly larger diameter after complexation as compared to AEMA–MMA particles alone. This can affect cellular uptake of the particles. Also, AEMA–MMA particles lose their positive charge upon complexation affecting their interaction with the negatively charged cell membrane. This phenomena is not as significant for AEMA–PEGMA particles, the platinum-complexed AEMA–PEGMA particles are small and also retain significant positive charge, even after complexation. Hence, these particles show more toxicity to cells.

Both of the particulate systems interact with the cells and have a high LC_{50}, showing that they are only mildly cytotoxic to the cells. From the available data, we cannot conclusively say if these particles are endocytosed by the cells. However, their interaction with the cell membrane may allow the release of the drug into the cells either by endocytosis or local drug release. The fact that the cytotoxicity of these particles is low makes them a good vehicle to be explored for small molecule or DNA delivery. AEMA–PEGMA particles may be especially useful, since they retain some of their positive charge and do not show high cross-linking after complexation and, hence, are able to show toxicity towards the glioma cells.

The use of many potent anticancer drugs has been limited because of systemic toxicity and inability to cross the blood–brain barrier. With the ability of the particles to associate with cells, the drug may be released in the proximity of the cells, thus reducing toxicity arising from unnecessary exposure of free drug to unaffected organs. The release profiles of both particulate systems are similar, except in the first phase. Both particles show a sustained release. This is due to the fact that the binding of the platinum to the amino groups on the particles is strong. The release of the drug may be manipulated by incorporation of a biodegradable linker which would be cleaved under certain circumstances, releasing the drug locally at the desired site [27]. The use of biodegradable linkers for selective drug release has been successfully studied using various drugs and polymers [28]. These particles show good potential for such a design due to the presence of amino groups for attachment. Due to their lower toxicity and cationic charge of AEMA–PEGMA,

these particles may be able to successfully deliver many types of drugs. This also opens up opportunities to drugs which may be potent but are not used due to lack of an efficient delivery system.

CONCLUSIONS

AEMA–MMA and AEMA–PEGMA particles have shown good physical properties for use as drug-delivery systems. AEMA–PEGMA particles may be more suitable for drug delivery for the various reasons discussed. The size may be appropriate for intratumoral delivery of drugs, although further studies are necessary to show *in vivo* distribution and kinetics. The number of amino groups could result in high drug loading and, therefore, could reduce the drug dose administered. The high payload with prolonged release reduces the frequency of administration. This may be especially advantageous for local administration with reduced systemic toxicity. The particles alone were only mildly cytotoxic and associated with tumor cells. These particles have a very good potential for drug delivery, targeted drug delivery and gene therapy, and should be explored further for such applications.

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