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Synthesis, characterization and *in vitro* cytotoxicity evaluation of polyamidoamine conjugate containing pamidronate and platinum drug

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ABSTRACT

Bisphosphonates have been found to be effective when combined with anticancer drugs for chemotherapy. In this paper, pamidronate and platinum complexes were conjugated to linear poly(amidoamine)s (PAMAM) to improve the drug efficacy. The conjugates were synthesized by aqueous phase Michael-addition polymerization reaction and characterized using SEM, TEM, XRD, FTIR, NMR and EDS to confirm successful conjugation of the drug to the polymeric carrier. *In vitro* cytotoxicity assays were performed against HeLa cell lines. FTIR, NMR and EDS confirmed the conjugation of the drugs to the polymer, and viability assay confirmed that the conjugates were not as toxic as the free drugs to the cells. The results obtained suggest that PAMAM are potential drug delivery devices for anticancer drugs with enhanced therapeutic effects. However, further characterization and *in vitro* tests will need to be conducted before further steps are taken.

1. Introduction

Breast cancer is the leading cause of cancer-related mortality among women. In 2012, 1.7 million new cases were diagnosed, representing about 12% of all new cancer cases and 25% of all cancers in women [1]. The most target site for metastasis in breast cancer is the bone [2,3]. Reports have shown that over 70% of women with advanced stage of breast cancer suffer from bone metastasis resulting in severe pain, spinal compression and hypercalcemia [2–4]. Combination chemotherapy is an effective treatment option for the management of cancer. Combining two drugs with different mechanisms of action trigger synergistic effects than the individual drug. The classes of drugs used for treatment of breast cancer are; alkylating agents, antracyclines, platinum-based drugs, taxanes and bisphosphonates *etc*.

Bisphosphonates used for the treatment of bone resorption have been reported to be effective when combined with antineoplastic drugs to treat breast cancer that has metastasised to the bone [3,5–8]. They exhibit a high binding affinity for hydroxyapatite in the bone and they act directly on osteoclasts [5,9]. They decrease the lifespan of matured osteoclasts by apoptosis thereby inhibiting proliferation and differentiation of pre-osteoclasts [6,7]. Some research reports have also shown that bisphosphonates exhibit cytostatic effects on several human cancer cell lines [10–12]. Bisphosphonates delay progression of bone disease.

Some platinum-based drugs which have been developed with anticancer effects are: cisplatin, carboplatin, oxaliplatin, laboplatin *etc*. Cisplatin is the most used drug for the treatment of all forms of breast cancer. The general mechanism of action of platinum complexes is believed to involve the formation of aquated species upon administration followed by subsequent intra- and inter-strand crosslinking with intracellular DNA, resulting in irreversible lesions in the double helix and ultimate cell death [13–15]. Despite their effective use in the treatment of cancer, they suffer from severe pharmacological shortcomings such as drug resistance, lack of cell selectivity whereby both the healthy cells and organs are exposed to the toxic side effects such as nephrotoxicity, neurotoxicity and ototoxicity [16,17].

Combining bisphosphonates with anticancer drugs such as platinum drugs have been reported to be effective for the treatment of breast cancer. Incorporating drugs onto polymer offers several advantage such as: improved drug pharmacokinetics which result in increased efficacy of the drug; improved drug solubility, enhanced drug bioavailability, improved drug targeting and release mechanisms [18–22]. There are

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various polymers that are being explored by scientists, however this research focuses only on linear poly (amidoamine)s (PAMAM). PAMAM are pH sensitive, water-soluble, stable at physiological pH, exhibit low toxicity, flexible, their rate of hydrolysis can be controlled by the nature of degradable linkage used and they are suitable as drug delivery systems [23]. Due to the above mentioned properties of PAMAM, they are therefore ideal drug delivery systems for targeted delivery of anticancer drugs. In this research, PAMAM were synthesized by a one-step Michael addition polymerization reaction followed by characterization using Fourier-Transform Infrared (FTIR), Scanning Electron Microscope (SEM), Nuclear Magnetic Resonance Spectroscopy (NMR), Energy-Dispersive X-ray Spectroscopy (EDS) and in-vitro cytotoxicity assay of PAMAM conjugates.

2. Experimentals

2.1. Materials

The following reagents were purchased from Sigma Aldrich, South Africa: phosphorous acid, phosphorous trichloride, dopeamine hydrochloride, *N,N´*-methylene bisacrylamide (MBA), *N,N′*-Dimethyl-1,3-propanediamine (DEP), triethylamine (TEA), deuterium Oxide (D_2O). Methane sulfonic acid was obtained from (Merck Chemicals, South Africa). Distilled water was used for the Michael addition polymerization reaction and dialysis. Dialysis was performed using cellulose membrane spectra (12000–14000 molecular mass cut off) from Spectrum Industries, Los Angeles, CA. [Pt(1R,2R-diaminocyclohexane)$(H_2O)_2$]$(NO_3)_2$ was purchased from (Kunming Institute of Precious Metals, China). Dulbeco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and pen/strep antibiotic were purchased from (Gibco, Invitrogen, Paisley, UK). LOT: 01. Minimum essential medium (MEM) was purchased from (Separations, South Africa) and human insulin (0.5 mg/ml) was purchased from (Biochrom GmbH, Berlin, Germany). HeLa cell lines were obtained courtesy of CSIR Pretoria, Polymers and Composites Department.

2.2. Synthesis of pamidronate

Pamidronate was synthesized according to the method by Kieczykowski et al. [24]. NMR analysis was performed using D_2O. ^{31}P NMR spectrum showed peak at 16.99 ppm. ^{1}H NMR spectrum displayed two peaks between 1.98-2.06 and 3.09–3.12 ppm, respectively for $NH_2\mathbf{CH_2}$ and $\mathbf{CH_2}COH$ confirming the successful isolation of the compound [24,25].

2.3. Preparation of polymer-drug conjugates

The polymer-drug conjugates were prepared from poly(amidoamines) polymers by aqueous Michael addition polymerization reaction (Table 1, Fig. 1). They conjugates were prepared using method earlier reported by our research group [26]. The conjugates were characterized by ^{1}H and ^{31}P NMR spectroscopy (Table 2).

2.3.1. PAMAM-Pt-Pam conjugate

The conjugate was prepared by dissolving MBA in warm distilled water (10 mL). DEP was added followed by continuous stirring at room temperature for 2 h before the addition of pamidronate and triethylamine (0.5 mL), respectively. The resultant solution was flushed with nitrogen gas and stirred overnight at room temperature. Dopamine was added and the resultant solution was stirred at room temperature overnight. Platinum (II) complex, diaminocyclohexaneplatinum(II)nitrate was added and the pH was maintained at 5.5–6.0 using hydrochloric acid. The resultant solution was protected from light, flushed with nitrogen gas and stirred at room temperature for 5 days. Conventional work up process was performed by filtering the resultant solution, performing dialysis on the filtrate against water using cellulose membrane with a molecular mass cut off 12000–14000 followed by freeze-drying. Brown water-soluble solids were isolated (0.30 g, 40% yield).

2.3.2. PAMAM (carrier)

MBA was dissolved in 10 mL of distilled water followed by the addition of DEP. The resultant solution flushed with nitrogen gas and then stirred at room temperature for 1 h. Dopamine was then added and reaction was protected from light using aluminium foil and reaction was stirred at room temperature for 3 days. The conventional work-up process used involved adjusting the pH to 7–8 with concentrated hydrochloric acid followed by exhaustive dialysis against water using cellulose membrane with a molecular mass cut off 12000–14000 and freeze-drying. White water-soluble solids were isolated (0.28 g, 45% yield).

2.3.3. PAMAM-Pam conjugate

The conjugate was prepared using the aforementioned method by dissolving MBA in 10 mL of distilled water followed by the addition of pamidronate and triethylamine, respectively. DEP was then added and the resultant solution was stirred at room temperature for 3 days. The conventional work up process was employed and white water-soluble solids were isolated (0.30 g, 49.5% yield).

2.3.4. PAMAM-Pt conjugate

Using the aforementioned method, MBA was dissolved in 10 mL of distilled water followed by the addition of DEP with continuous stirring at room temperature for 2 h. Dopeamine and triethylamine were then added followed by continuous stirring at room temperature overnight. Platinum (II) complex, diaminocyclohexaneplatinum (II) nitrate and the pH was maintained at 5.5–6.0 using concentrated hydrochloric acid. The resultant solution was protected from light, flushed with nitrogen gas and stirred at room temperature for 5 days. Conventional work up process was performed as indicated in section 2.4.1. and brown water-soluble solids were isolated (0.30 g, yield 40%).

2.4. Characterization

2.4.1. FTIR

The FTIR analysis was performed within the range of 4000–500 cm^{-1} on a Perkin Elmer Spectrum 100 FTIR spectrometer (Waltham, MA).

2.4.2. SEM and EDS

The surface morphologies of the conjugates, carrier and free drug were evaluated using SEM. The elemental composition of the carriers

Table 1
Quantity of reagents used.

Conjugates/Carrier	MBA	DEP	Pt (II)	TEA	Dopamine	Pamidronate
PMAMA-Pt-Pam	300 mg, 1.95 mmol	152 mg, 1.17 mmol	183 mg, 0.39 mmol	0.5 mL	74 mg, 0.39 mmol	92 mg, 0.39 mmol
PAMAM	300 mg, 1.95 mmol	203 mg, 1.56 mmol	–	0.5 mL	74 mg, 0.39 mmol	–
PAMAM-Pam	300 mg, 1.95 mmol	203 mg, 1.56 mmol	–	0.5 mL	–	92 mg, 0.39 mmol
PAMAM-Pt	300 mg, 1.95 mmol	203 mg, 1.56 mmol	183 mg, 0.39 mmol	0.5 mL	74 mg, 0.39 mmol	–