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Cellular internalisation kinetics and cytotoxic properties of statistically designed and optimised neo-geometric copper nanocrystals

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ABSTRACT

This study aimed to highlight a statistic design to precisely engineer homogenous geometric copper nanoparticles (CuNPs) for enhanced intracellular drug delivery as a function of geometrical structure. CuNPs with a dual functionality comprising geometric attributes for enhanced cell uptake and exerting cytotoxic activity on proliferating cells were synthesized as a novel drug delivery system. This paper investigated the defined concentrations of two key surfactants used in the reaction to mutually control and manipulate nano-shape and optimisation of the geometric nanosystems. A statistical experimental design comprising a full factorial model served as a refining factor to achieve homogenous geometric nanoparticles using a one-pot method for the systematic optimisation of the geometric CuNPs. Shapes of the nanoparticles were investigated to determine the result of the surfactant variation as the aim of the study and zeta potential was studied to ensure the stability of the system and establish a nanosystem of low aggregation potential. After optimisation of the nano-shapes, extensive cellular internalisation studies were conducted to elucidate the effect of geometric CuNPs on uptake rates, in addition to the vital toxicity assays to further understand the cellular effect of geometric CuNPs as a drug delivery system. In addition to geometry; volume, surface area, orientation to the cell membrane and colloidal stability is also addressed. The outcomes of the study demonstrated the success of homogenous geometric NP formation, in addition to a stable surface charge. The findings of the study can be utilized for the development of a drug delivery system for promoted cellular internalisation and effective drug delivery.

1. Introduction

Surface engineering of nanotherapeutics is considered as the frontrunner in biomedical applications and drug delivery as a smart means of delivering drugs and dictating their fate. Nanoparticles (NPs) as a drug carrier have been exploited in the delivery of biomedical agents owing to its nano-size allowing for drugs to be delivered at a cellular level thereby increasing drug deposition intracellularly, reducing the amount of drug required and more importantly, reducing the side effects of drugs by targeting NPs to diseased cells. With this emerged field of research gaining significant momentum since its inception, the pathway has been paved for improvements on the original concept of NPs as a drug delivery system.

With recent advances in nanotechnology, studies show that manipulating the physicochemical and physicomechanical parameters of NPs have an effect on the cellular internalisation [1,2]. Modifying the characteristics of NPs assist with the enhanced internalisation and improving therapeutic time. Engineering nano features such as size, shape, surface charge, chemical chemistry, hydrophobicity and ligand attachments are a few parameters of nanoconstructs that can be manipulated [3,4]. If focused on during synthesis, these characteristics may be the dictating factor in improving NP internalisation kinetics, thus, positively affecting intracellular drug delivery.

Among these possible NPs modifications, NPs with engineered geometries seem to be an interesting one with varying rates of internalisation based on the interactions between the scaffold design and cell membrane. Nano-shape and geometry-related parameters form factors inclusive of aspect ratios or edges that affect NP internalisation kinetics and influence cell-particle interactions [5,6]. By varying the shape of NPs having the same volume, or varying the volume of particles of the same shape, it can be studied that NPs with different shapes penetrate the lipid bilayer of the cell membrane differently and geometric internalisation may even vary between different cell types [6]. Nano-vector design as a function of its shape has been proven to have diverging effects on cell uptake which also ranges from active and passive translocation mechanisms such as phagocytosis, macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis [3].

Hence, the aim of the study was to engineer neo-geometric CuNPs in the investigation of nano-shape in drug delivery application. As for any formulation, design criteria including material selection and network

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fabrication are crucial for drug delivery. In this study, these criteria are essential in governing the outcome of the geometrical structure and charge stability of the CuNPs. Prior to the synthesis of the CuNPs, these criteria have to be evaluated based on the two independent variables. In order to achieve the aim of formulating homogenous, stable CuNPs, extensive shape elucidating characterisation and zeta potential analysing were conducted. The use of the two key surfactants offered shape dictation and varying surface charge of the formulations in the statistical design for its use in further studies to investigate the effect of shape on cell uptake, simultaneously improving drug delivery. As a result, in this investigation a new strategy for the preparation of novel geometric CuNPs, with optimisation through a full factorial experimental design is reported on.

Based on the concept of nano-geometry influencing cell uptake, after optimisation of the nano-systems, this study investigated the internalisation kinetics of neo-geometric CuNPs on normal human epidermal keratinocytes (NHEK) and cytotoxic analyses using NHEK and human cervical cancer cells (HELA). The successful optimisation of the bottom-up synthesis of geometric CuNPs synthesized by selective surfactant-mediated surface adsorption on preferential crystal facets serves as the geometric nano-vector.

2. Materials and methods

Copper II sulphate pentahydrate ($CuSO_4 \cdot 5H_2O$) was purchased from Merck (Darmstadt, Germany), CTAB, SDS and MTT, LDA, GSH and MDA assays were purchased from Sigma-Aldrich Co. (Aldrich, Steinheim, Germany) and L-ascorbic acid (98%) was purchased from Roche (Johannesburg, South Africa). Normal human epidermal keratinocytes (NHEK), keratinocyte basal medium, growth factors and supplements including bovine pituitary extract, human epidermal growth factor, insulin, hydrocortisone, epinephrine, gentamicin, amphotericin B, transferrin, trypsin/EDTA were all purchased from Whitehead Scientific (Cape Town, South Africa). HELA cells and Dulbecco's Modified Eagles Medium were purchased from Separations (Johannesburg, South Africa). All chemicals and assay kits were analytical grade and used without further purification.

2.1. Synthesis of geometric copper nanocrystals

The thermal-reduction method of synthesizing nanocrystals included a standard $CuSO_4 \cdot 5H_2O$ solution added to varied molar concentrations of aliquot hexadecetyl trimethylammonium bromide (CTAB) and sodium dodecyl sulphate (SDS) solutions heated at 50 °C. Solution was further heated between 85 and 90 °C and a standard ascorbic acid solution was added as the reducing agent in a drop-wise manner to allow for spontaneous copper formation without a reducing agent overload. The temperature was then maintained at 80 °C using a mercury thermometer (Brannon Thermometers, Cumbria, England).

2.2. Experimental design and constraint optimisation of neogeometric copper nanoparticles

A full factorial design was used to optimise the neogeometric nanoparticles. Optimisation using the two variable design model was employed to ascertain the ideal combination of dual surfactants (CTAB and SDS) as the independent variables to achieve optimal homogenous geometries and zeta potential to ensure stable nanosystems. For each of the two parameters selected, two factors were fixed, an upper and a lower level as summarized in Table 1 and these values were obtained from preliminary studies undertaken with 4 levels for CTAB and 3 levels for SDS. Also shown in Table 1 are the responses and the optimisation constraints for each variable. The design was both generated and analysed using Minitab V15 software (Minitab® Inc., PA, USA) and for the design 12 experimental formulations were obtained as summarized in Table 2.

Table 1
Formulation variables and responses applied in the full factorial design.

	Levels		Objective
	Upper	Lower	
Parameters			
CTAB (M)	0.040	0.000	
SDS (M)	0.100	0.000	
Responses			
Shape uniformity			Maximise
Surface charge			≤25≥

2.3. Geometric and morphological characterisation of the copper nanoparticles

TEM (FEI T12 Spirit TEM (120 kV), Hillsborough, USA) and High-Resolution TEM (JEOL JEM 2100F (200 kV), Oregon, USA) were conducted to confirm the synthesis of self-assembled neogoemetrical NPs. CuNPs were dispersed on a copper grid coated with Formvar/carbon (200 mesh, BAL-TEC, EMTechnology and Application, Witten, Germany). Excess sample was removed by solvent evaporation under ambient conditions. Samples were also subjected to TEM-EDS for high-speed elemental analysis.

2.4. Zeta potential and stability analyses of copper nanoparticles

The zeta potential measured the surface charge of the CuNPs, thus indicating the stability and aggregation potential of the NPs. Each suspension was diluted 1:15 in distilled water, filtered through a 0.22 μm millipore filter, transferred into a capillary cell and was analysed by a Zeta sizer (DTS (nano), Malvern instruments ltd, Worcestershire, UK).

2.5. Crystal and elemental analysis of the optimised copper nanoparticles

A powder X-Ray diffractometer (MiniFlex 600, Tokyo, Japan) was used to monitor diffraction patterns of the CuNP samples to validate the synthesis of crystalline copper. A continuous scan rate of 0.1°/min from 0 to 90° was used with Cu Kα radiation ($\lambda = 1.54$ Å). Percent crystallinity of distinctive peaks was computed employing PDXL software (Rigaku, Tokyo, Japan). Concurrent characterisation was carried out using an Oxford INCA Energy Dispersive Spectroscopy (EDS) system coupled to a TEM which monitored diffraction patterns to determine the elemental composition of the CuNP samples.

2.6. Thermal degradation analysis of the surface polymer-coating of the copper nanoparticles

Thermogravimetric analysis (TGA) using a 4000 thermogravimetric analyzer (PerkinElmer Inc., Massachusetts, USA) evaluated the

Table 2
Statistically generated formulations obtained from a full factorial design of optimisation.

Formulation	Variable 1 [CTAB] (M)	Variable 2 [SDS] (M)
1	0.04	0.000
2	0.00	0.087
3	0.02	0.000
4	0.04	0.100
5	0.00	0.100
6	0.00	0.000
7	0.02	0.100
8	0.04	0.087
9	0.02	0.087
10	0.03	0.000
11	0.03	0.087
12	0.03	0.100