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Hollow microspheres based on – Folic acid modified – Hydroxypropyl Cellulose and synthetic multi-responsive bio-copolymer for targeted cancer therapy: Controlled release of daunorubicin, in vitro and *in vivo* studies

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ABSTRACT

Hypothesis: Conventional chemotherapy drugs such as anthracyclines show no specific activity. They destroy cancer cells but also and the healthy ones, and for that reason exhibit high toxicity. In order to alleviate the toxic effects of chemotherapeutic drugs, the administration dose is being minimized, while their reactivity against tumor cells is lessened. This problem can be overcome or at least reduced by using nanoscale drug delivery systems to target the pathogenic area. The present work deals with the synthesis, characterization and biological evaluation of multi-responsive hollow microspheres coated with Hydroxypropyl Cellulose (HPC)-a biocompatible and thermosensitive polysaccharide-conjugated with folic acid as well promising drug vehicles for targeted cancer therapy.

Experiments: The synthetic route consists of two steps. In the first step, a single layer of sensitive copolymers is ((Methacrylic acid (MAA), N-(2-Hydroxypropyl) methacrylamide (HPMA) and N,N′-(disulfanediylbis(ethane-2,1-diyl))bis(2-methylacrylamide) (DSBMA)) fabricated on a sacrificial template of SiO_2 and in the second step, an additional layer of the folic acid modified HPC coat the microspheres' surface. The layers fabrication is performed through a combination of distillation precipitation co-polymerization and chemical deposition method. The loading capacity (% LC) and encapsulation efficiency (% EE) percentages of the chemotherapeutic agent daunorubicin (DNR) in the fabricated microspheres were calculated through the standard curve methodology. In addition, the releasing properties of the resulting spheres are investigated, using the above mentioned methodology. It is worth mentioning that, spheres release the entrapped drug under combined conditions such acidic and reductive environment along with conventional hyperthermia. Cytotoxic activity of the synthesized spheres was investigated by using the well-established method of MTT assay in MCF-7 (breast cancer), HeLa (cervical cancer) and HEK 293 (Human Embryonic Kidney healthy cells) cell lines. Confocal and fluorescence microscopy were used to confirm the in vitro targeted ability of folic acid modified drug loaded microspheres in HeLa, to that overexpress folate receptors, MCF-7 and 3T3 cells, as negative folate cell substrate. Finally, radiolabelling of the spheres is performed, with a gamma emitting radionuclide (^{99m}Tc), to assess their *in vivo* profile by means of scintigraphic imaging and biodistribution studies.

Findings: Hollow spheres release the encapsulated drug under acidic environment, conventional hyperthermia or in the presence of glutathione (reductive environment). The ability of modified drug carriers to target the HeLa cells, was confirmed by confocal and fluorescence microscopy. The resulting spheres are observed to be promising drug-carriers for cancer treatment due to their releasing properties under tumor's environment and high concentration in HeLa cells *via* endocytosis. In addition, the empty vehicles have no toxicity in healthy cells and present antimicrobial activity.

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1. Introduction

Despite the efforts made in last years on antitumor drug discovery, conventional chemotherapeutic agents still show up poor specificity in tumor area and high toxicity [1]. Taking into consideration the above mentioned unsolved problems, recent interest has been focused on developing nanoscale delivery systems which regulate the release of chemotherapeutic drugs directly inside the cancer cells. This kind of delivery systems may have various attributes: (1) biocompatibility and biodegradability, (2) stay for a long in the body (3) high stability in the blood circulation, (4) improvement of drug solubility (3) stimulus response in relation to particular tumor characteristics, such as abnormal pH values (usually acidic pH), high temperature and reductive environment (5) specific targeting of therapeutic agents to the pathogenic area using ligands or antibodies [2,3–6].

According to literature, polysaccharide nanostructures could prolong the residence time in human body and increase the absorbance and solubility of loaded drugs. As natural materials polysaccharides have important advantages such as increased stability, safety, hydrophilicity, biocompatibility and reduced or existent toxicity. In addition, polysaccharides have abundant resources in nature and low cost effective for their production. Nevertheless, the potential of polysaccharides drug carriers is still under estimated and deserves more attention [6–8].

Cellulose, a promising representative of polysaccharides, is a linear homopolymer consisting of region- and enantioselective β-1,4 glycosidic linked D-glucose units and is the most common organic polymer in earth [9]. Due to its unique properties such as biocompatibility and anti-microbial action, it is one of the most promising materials for bio-applications. Although it is well known that cellulose is insoluble in the most common organic solvents and water, because of the strong intra and inter-chain molecular hydrogen-bonding interactions between hydroxyl groups and oxygen of the glucose's ring, can be chemically modified for improving their solubility in water. There are several commercial soluble derivatives of cellulose (cellulosics) such as Methylcellulose (MC), Hydroxyethyl Cellulose (HEC), Hydroxypropyl Cellulose (HPC) and Hydroxypropyl methylcellulose [10].

Although that last years, there is an increase of the publications related to delivery systems based on polysaccharides, a few of them focus on cellulose-based drug delivery systems and more particularly on drug formulations for specific diseases and in-vitro/*in-vivo* applications [11].

In our work, it was used HPC, a non-ionic, thermoresponsive and pH insensitive biodegradable polysaccharide, as the desired derivative of cellulose. It is well known that the HPC low critical solution temperature in water (LCST) is at 41 °C [12]. Based on that, HPC was modified by covalently attaching folic acid for targeting purposes. Folic acid (vitamin B9) is used for selective cancer cells targeting utilizing the folate receptor for active targeting of specific cancer cells (FR). FR is scarcely expressed in normal cells but it is overexpressed in a range of cancers such as ovary, brain, kidney, breast, myeloid cells, lung and cervical cancer [13]. The small size of folic acid (1 nm) and the high chemical affinity with their receptor (FR), allows the modified drug carriers endocytosis [14,15]. Many scientific groups exploit the tumor specific characteristics aiming at designing advanced drug delivery systems. The advantage of this strategy is that despite the systemic administration of the nanoparticle-mediated drug delivery, localized drug release will be accomplished. To achieve this goal, in the frame of the present synthetic process, there were used polymers that their behavior or their structure is affected by different conditions [2,16].

More specifically, inside cancer cells the concentration of glutathione is increased compared to its extracellular concentration [2,17]. For this reason we used as a crosslinker of the synthesized co-polymeric layer, the monomer (N,N′-(disulfanediylbis(ethane-2,1-diyl))bis(2-methylacrylamide) (DSBMA), which is sensitive in reductive environment due to the disulfide bond [2]. In the presence of glutathione inside the pathogenic cell the disulfide bridges of the polymeric layer are transformed into thiol groups, resulting in the slow release of daunorubicin [2].

Polymers consisting of N-(2-Hydroxypropyl) methacrylamide (HPMA) have been widely used in drug delivery systems for targeting tumor cells due to their input to the biodistribution profile of therapeutic agents and to prolongation of circulation time in the body. According to published research findings, HPMA-based polymers improve the diffusion of polymeric systems between the gaps of epithelial cells and their accumulation in the tumor *via* EPR effect (Enhanced permeability and retention effect). Even if HPMA's use is common in the field of linear or grafted copolymers, only a few examples of HPMA's have been reported in microspheres' fabrication related literature [18].

Based on the above, according to literature, folic acid modification is a well-established methodology to investigate the in-vitro nanotechnology based materials uptake that can be used for *in-vivo* applications as well. In the frame of this work, the spheres have been modified by folic acid for specific targeting of cancer cells in order that after being internalized, the polymeric network collapses and releases the drug inside the cells.

2. Material and methods

2.1. Materials

Hydroxypropyl Cellulose (HPC), MW = 350.000 g/mol, was purchased from Aldrich. 2,2-azobis(2-methylpropionitrile) (AIBN), N-hydroxysuccinimide (NHS), 1-ethyl-(3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 3-methacryloxypropyltrimethoxysilane (MPS) were purchased from Acros Organics and used as received. Ethylene glycol dimethacrylate (EGDMA) was purchased from Sigma Aldrich. Tetraethyl orthosilicate (TEOS) was purchased from Fluka. Methacrylic acid (MAA) was purified by distillation before using and acetonitrile was used as received from Aldrich. Methacryloyl chloride (97%) and 1-amino-2-propanol was purchased from Alpha. Daunorubicin HCl (DNR) was provided by Pharmacia & Upjohn and used as received. Fetal Bovine Serum (FBS) was purchased from Biochrome. High glucose Dulbecco's modified Eagle Medium (DMEM) and MTT were purchased from Sigma. Trypsin-EDTA, L-glutamine, penicillin–streptomycin solution were obtained from Biochrom KG, Berlin, Germany. Technetium-99m was used as a $Na^{99m}TcO_4$ solution in saline, eluted from a commercial Mallinckrodt Medical B.V. ^{99}Mo–^{99m}Tc generator.

2.2. Statistic analysis

The Origin 8.0 was used to analyze the data. The stantar curves were performed using Microsoft Excel.

2.3. Synthesis of organic molecules

2.3.1. Synthesis of N,N′-(disulfanediylbis(ethane-2,1-diyl))bis(2-methylacrylamide), (DSBMA)

DSBMA was synthesized *via* nucleophilic substitution of methacryloyl chloride from cystamine dihydrochloride. 5 g (0.022 mol, 1 eq) cystamine dihydrochloride was dissolved in water (25 ml H_2O) under magnetic stirring for several minutes. 3.52 mg (0.088 mol, 4 eq) NaOH was cooled at 0 °C and then added to the