Antitumor Agents

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Acylsulfonamide-Functionalized Zwitterionic Gold Nanoparticles for Enhanced Cellular Uptake at Tumor pH**

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Abstract: *A nanoparticle design featuring pH-responsive alkoxyphenyl acylsulfonamide ligands is reported herein. As a result of ligand structure, this nanoparticle is neutral at pH 7.4, becoming positively charged at tumor pH (< 6.5). The particle uptake and cytotoxicity increase over this pH range. This pH-controlled uptake and toxicity makes this particle a promising tool for tumor selective therapy.*

The pH difference between normal tissue (pH 7.2–7.4) and tumor tissue (pH 6.0–6.8)[1] provides an attractive strategy for selective accumulation of nanoparticles (NPs) into tumor tissues for cancer treatment and/or imaging. To supply high tumor selectivity, a pH-responsive bio-interactive surface must be generated with appropriate responses at normal and tumor pH values.[2] The most common strategy is the use of a non-interactive functional group bearing an acid-cleavable unit; however, difficulty in controlling the acid sensitivity can result in the cleavage reaction taking place even at neutral pH,[3] or requiring quite a low pH value (ca. 5.0) for activation.[4] The use of reversible protonation/deprotonation systems provides an alternate strategy that features high tunability.[5] Reports employing polymers, such as polyhistidine and polyamines, as pH-responsive moieties provided high tumor pH sensitivity.[6] Solubility can be a challenge, however, with these neutral-to-cationic systems, requiring the use of additional functional groups such as poly(ethylene)glycol chains at neutral pH.

Zwitterionic surfaces have recently received considerable attention as non-interacting chemical surfaces.[7] Nanoparticles with zwitterionic surfaces exhibit a long circulatory half-life,[8] low cytotoxicity,[9] and high biocompatibility. Integration of these properties with a pH-dependent zwitterionic-to-cationic charge conversion system would make them attractive scaffolds for therapeutics. The low cellular uptake of zwitterionic particles[10] makes them excellent candidates for pH-controlled tumor uptake upon protonation to the resulting cationic particle. Moreover, concomitant cytotoxicity resulting from these cationic NPs[11] would occur only in the tumor environment, leading to the possibility of tumor-selective therapy.

A few examples of pH-responsive zwitterionic chemical surfaces such as carboxybetaine[12] and phosphorylcholine have been reported.[13] However, the charge-switching abilities of these structures are not sensitive enough to respond to stimuli such as weakly acidic tumor pH, with carboxybetaines protonated at pH < 2 and phosphorylcholine at pH < 5. The structure of the negatively charged group is the key for precise pH responsiveness, and has been reported using mixed-monolayer particles featuring carboxylic acid and ammonium ligands.[14] Herein, we report a new pH-responsive zwitterionic surface structure engineered by derivatization of the acylsulfonamide group. This designed zwitterionic group becomes cationic at tumor pH (< 6.5), resulting in dramatically enhanced cellular uptake and cytotoxicity. Significantly, these particles show no hemolytic activity at the pH value of blood (pH = 7.4).

The acylsulfonamide group features behavior similar to that of carboxylic acid groups with changes in pH value.[15] We chose this group as the negatively charged part of the zwitterionic ligand, with pH responsiveness controlled by the functional group attached to the sulfonyl group. AuNP **1** features an aryl acylsulfonamide while AuNP **2** provides an alkyl analogue (Figure 1). The ligands have trimethylammonium termini as well as a tetra(ethylene glycol) spacer between the negative group and the hydrophobic alkyl chain. Tetra(ethylene glycol) spacer was used as a passivating group to avoid irreversible adsorption and denaturation of serum proteins.[16] These particles were synthesized from pentanethiol-capped AuNPs (ca. 2 nm core) by means of place-exchange reactions.[17]

We initially measured the zeta potential to determine the pH dependence of the AuNP surface charges (Figure 2). The surface charges of both AuNP **1** and **2** were close to neutral at physiological pH (7.4), consistent with the zwitterionic structure of these NPs. AuNP **1** features a sharp transition from neutral to cationic centered at pH 6.5, with consistent changes detected by ^{1}H NMR spectroscopy (see the Supporting Information, Figure S1).[18] In contrast, AuNP **2** displayed a zwitterionic surface even at acidic pH values, consistent with the reported pK_a value of an alkyl acylsulfonamide group

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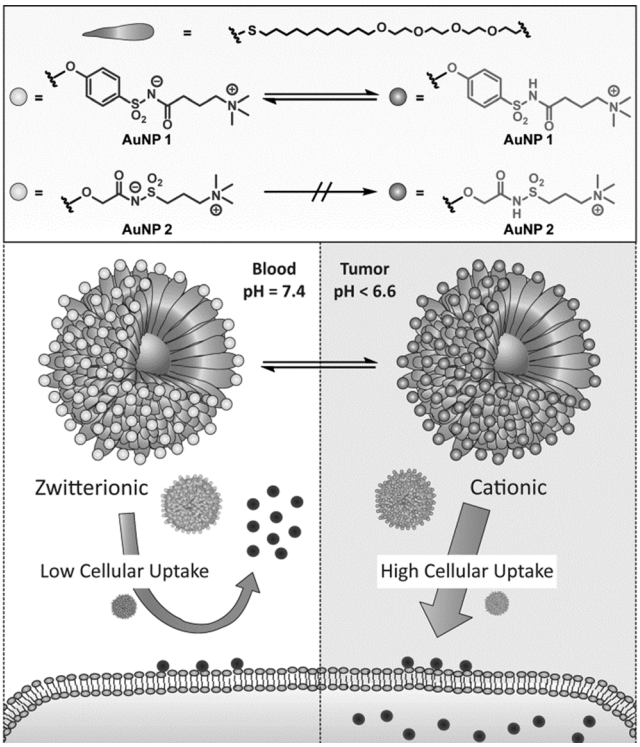

Figure 1. Chemical structure of the monolayer-protected gold nanoparticles (AuNPs) and our strategy for a pH-responsive delivery system into tumors.

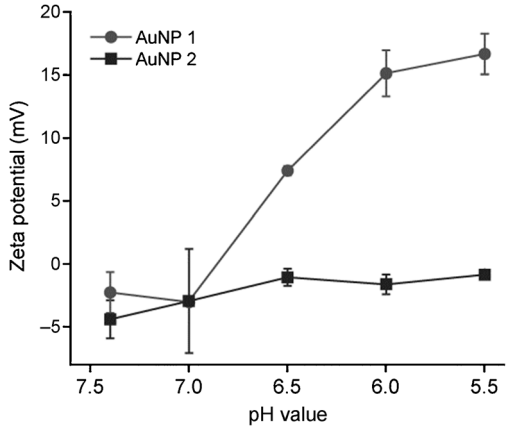

Figure 2. Zeta potential plotted against pH values for AuNPs **1** and **2** (both 1 μM). Zeta potentials were measured in phosphate buffer (5 mM) at different pH values. Error bars represent standard deviations based on three independent measurements per pH value.

($pK_a \approx 4.5$)[19] and providing a permanently zwitterionic control particle for our studies. Zeta potentials for particles with higher coverage obtained by two exchange reactions were similar to those formed with only one exchange (Figures S2 and S3). Recent reports on a pH-responsive zwitterionic AuNP with a mixed monolayer of carboxylic acid and quaternary ammonium ligands showed that the neutralization of surface charge during the charge alteration process results in the formation of precipitates.[14,20] Therefore, we investigated if similar aggregates formed in our case. Dynamic light scattering data revealed that no aggregation of these NPs was detected in the pH range from 7.4 to 6.0 (see the Supporting Information), preventing potential complications. The stability the particles to degradation in serum was confirmed by MALDI-MS (Figure S4) after the incubation in 10% serum containing media (37°C, 24 h).

The change of AuNP **1** from zwitterionic to a positively charged surface at a weakly acidic pH value should enhance cellular uptake of AuNPs. Both AuNPs **1** (switchable) and **2** (nonswitchable) were incubated with HeLa cells for 3 h at three different pH values,[21] with the resulting intracellular amount of gold measured using inductively coupled plasma mass spectrometry (ICP-MS; Figure 3). At pH 7.4, very low

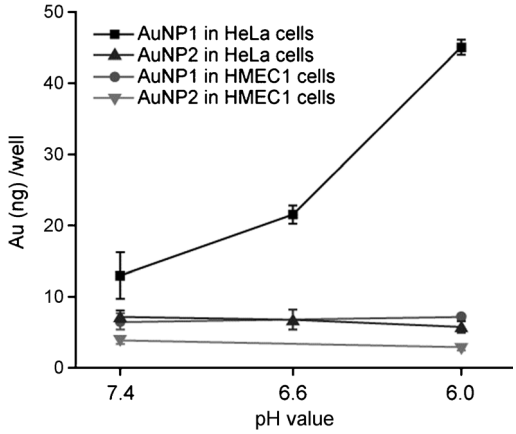

Figure 3. Cellular uptake of AuNPs **1** and **2** (both 1 μM) after 3 h incubation with HeLa (30000 cells/well) or HMEC-1 cells (100000 cells/well) in the presence of 10% serum. All experiments were performed in triplicate, and error bars represent standard error of the mean.

uptake (approximately 10 ng/well) was detected for both AuNPs **1** and **2**, similar to the cellular uptake of recently reported highly nonfouling sulfabetaine functionalized AuNPs.[7d] Uptake of AuNP **1** increased with decreasing pH value, reaching a fourfold increase at pH 6.0. In contrast, no significant change in uptake was observed with control AuNP **2** over this pH range. Transmission electron microscopy (TEM) was used to evaluate the intracellular localization of AuNPs at different pH values. AuNP **1** was located only on the cell membrane at pH 7.4, whereas endosomal uptake was observed at pH 6.0 (Figure S5), confirming that an acidic pH value leads to protonation and triggers concomitant endosomal uptake of AuNP **1** in HeLa cells. Taken together, these studies establish the role of acylsulfonamide protonation in determining cellular uptake.

We next investigated the pH-dependent cellular uptake in human microvascular endothelial cells (HMEC-1). At pH 7.4, the amount of intracellular gold was similar to that detected with HeLa cells. In contrast to HeLa cells, no significant difference in uptake was observed even at pH 6.0 for HMEC-1 cells (Figure 3). This is presumably due to differential uptake mechanisms in HeLa and HMEC-1 cells.[22] Significantly, these results indicate that the cationic charge on the

nanoparticle surface can provide different affinity and selective uptake in HeLa cells relative to HMEC-1 cells, which could be a critical parameter for selective delivery of nanomaterials in a tumor microenvironment.

Positively charged AuNPs show higher cytotoxicity than their zwitterionic counterpart,[10] which indicates that cytotoxicity of AuNP **1** could be triggered by a weakly acidic pH value. Therefore, we next evaluated the cytotoxicity of AuNPs **1** and **2** at varying pH values and concentrations using the Alamar blue viability assay (Figure 4). At pH 7.4, no

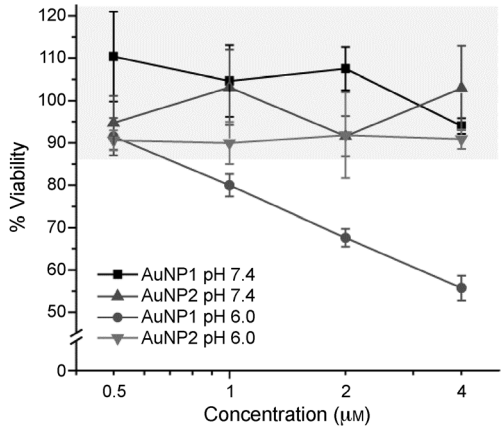

Figure 4. Cell viability of HeLa cells after 72 h incubation with AuNPs **1** and **2** (0.5–4.0 μM) in the presence of 10% serum. All experiments were performed in triplicate. Error bars are standard error of the mean.

cytotoxicity of AuNPs **1** and **2** was observed in HeLa cells as a result of their non-interactive zwitterionic properties. Enhanced cytotoxicity of AuNP **1** on HeLa cells was observed at pH 6.0, as expected because of the enhanced cellular uptake resulting from the conversion of the particle from zwitterionic into cationic. A glucose-6-phosphate dehydrogenase (G6PD) assay indicated no cell membrane damage (Figure S6), indicating that cytotoxicity arose from intracellular mechanisms, for example, oxidative stress or DNA damage.[11] In contrast, AuNP **2** did not show any significant toxicity at low pH values. In addition, no cytotoxicity was also observed in HMEC-1 cells at both pH 7.4 and pH 6.0 (Figure S7).

Hemolysis is an important issue for therapeutic nanomaterials.[23] Therefore, we performed a hemolytic assay to investigate the blood compatibility of our NPs. AuNP-mediated cell-lysis events were not observed on either AuNP **1** or **2** in both the presence and absence of plasma proteins (Figures S8 and S9).[24]

In conclusion, we have developed a new pH-responsive zwitterionic ligand based on the alkoxyphenyl acylsulfonamide group. As a result of its precisely designed surface structure, zwitterionic AuNPs functionalized with this ligand reversibly become cationic at tumor pH, with concomitant enhancement of cellular uptake and cytotoxicity on HeLa cells. This combination of pH-regulated tumor-selective cellular uptake and cytotoxicity could make this ligand design promising for imaging, delivery, and self-therapeutic applications.

Keywords: antitumor agents · cellular uptake · gold · nanoparticles · zwitterionic ligands

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