Application of Crumpled Aluminum Hydroxide Nanostructures for Cancer Treatment

Aleksandr S. Lozhkomoev, Georgy Mikhaylov, Vito Turk, Boris Turk, and Olga Vasiljeva

Abstract The tumor microenvironment regulates tumor progression and the spread of cancer in the body. Applications of nanomaterials that can dysregulate tumor-microenvironment are emerging as a promising anti-cancer approaches, which can improve the efficacy of existing cancer treatments. We have reported that agglomerates of radially assembled Al hydroxide crumpled nanosheets with the disordered defective surface structure have a large positive charge and therefore can lead to ion imbalance at the cell perimembranous space through the selective adsorption of extracellular anionic species. This effect was demonstrated in vitro by reduced viability and proliferation of tumor cells, and further validated in a murine melanoma cancer model. Furthermore, crumpled Al hydroxide nanostructures showed a much stronger suppressive effect on tumor growth in combination with a minimally effective dose of doxorubicin. Taken together, the described approach of tumor microenvironment dysregulation through selective adsorption properties of folded crumpled nanostructures opened a new avenue for development of innovative anticancer therapy strategies.

Keywords Aluminum hydroxide · Nanosheets · Folded crumpled nanostructures · Tumor microenvironment · Anti-cancer therapy · Aloohene · Ion imbalance

G. Mikhaylov · V. Turk · B. Turk · O. Vasiljeva (✉)
Jozef Stefan Institute, 1000 Ljubljana, Slovenia
e-mail: olga.vasiljeva@ijs.si

A. S. Lozhkomoev
Institute of Strength Physics and Materials Science, Tomsk 634055, Russia

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1 Introduction to Low-Dimensional Aluminum (Hydro)oxides

Low-dimensional (2D) nanostructures have been extensively studied in the last 10–15 years [1–5]. The interest can be explained by the fact that two-dimensional nano-objects exhibit physicochemical properties significantly different from those of three-dimensional (bulk) materials. This is due to both the dominant role of the free surface and the morphological instability of the planar shape of 2D systems [6–9]. It should be noted that the current trend in the development of nanotechnology in the field of synthesis of low-dimensional nanostructures, possessing the necessary physicochemical and biomedical properties, is characterized by a broader use of the methods based on controlled self-organization (or self-assembling) of the structure directly in the synthesis process [10, 11]. In case of ultrathin 2D-nanostructures, the self-assembly of such ensembles is of high importance to fundamental and applied sciences, since it gives an opportunity not only to investigate a substance behavior in the two-dimensional state, but also to produce complex hybrid (organic-inorganic) nanosystems for a wide range of applications ranging from elements for sensor devices to nanostructures for biomedical applications [10, 12]. One of the promising directions in the formation of 2D nanostructures with a controlled structure is self-assembly during the oxidation of nanoparticles [10, 13].

The perspectives of new drugs development for the diagnostics and treatment of various diseases, including cancer, are associated with studies of low-dimensional nanostructures interaction with living cells [14–16]. The effects of colloidal gold particles [17], nanofibers or nanowires of zinc oxide [18, 19], titanium oxide [20], carbon nanotubes [21] and graphene oxide [22] have been the most studied as yet. Low-dimensional structures via self-assembling can form more complex ensembles [23]; however, the interaction of cells with such ensembles can be also studied at the level of individual low-dimensional components of the complex.

From the point of view of medical applications and targeted action on biological objects the low-dimensional aluminum (hydro)oxides should be considered as a promising basis for development of nanostructures with bio-activity. This is due to both their unique surface properties [12, 24], which determine their biological activity [25, 26], as well as their low toxicity [27] and the possibility to generate nanostructures with certain morphology during their synthesis [28].

2 Synthesis of Aluminum Oxyhydroxide Low-Dimensional Nanostructures

A simple and fast method for the synthesis of nanostructures based on the aluminum oxide and hydroxide phases is oxidation of aluminum nanopowder by water obtained via electrical dispersion of an aluminum wire [29, 30]. The synthesis proceeds in

a single stage at atmospheric pressure and a temperature of 50–70 °C. The reaction mixture contains only water and aluminum. Therefore, the reaction products do not contain organic and ionic contaminants. This is a major advancement from the previous studies, where oxidation of aluminum nanoparticles by water was studied only as a method for a hydrogen production, and much less attention was paid to the reaction products [31–36]. Nanoparticles of the aluminum nitride composition (Al/AlN) provide a better starting material for the reaction due to the presence of a thin oxide film on the surface of the particles and the release of ammonia during hydrolysis, which increases the pH of the reaction medium [37]. The oxidation process of Al/AlN nanoparticles proceeds without an induction period, due to a thinner oxide film, and is accompanied by complete oxidation of aluminum as a result of ammonia excretion during the reaction. In order to obtain the aluminum oxyhydroxide low-dimensional nanostructures, the AlN/Al nanopowder produced by electric explosion of an aluminum wire in a nitrogen atmosphere, was used in our research.

Nanopowder is represented by a mixture of spherical and faceted particles. According to the results of the elemental analysis, nitrogen is found in all particles, however it is distributed in spherical particles non-uniformly (Fig. 1a), indicating

Fig. 1 TEM image and EDS mapping (a), particle size distribution and XRD pattern (c) of AlN/Al nanoparticles

Fig. 2 TEM (a) and SEM (b) images of agglomerates of crumpled nanosheets. Reproduced with permission from ACS [38]

presence of metallic aluminum in them. The average particle size is 85 nm (Fig. 1b). According to the X-ray diffraction (XRD) analysis, the main peaks in the XRD pattern correspond to the Al and AlN phases (Fig. 1c).

As the result of AlN/Al nanoparticles oxidation in water at 60 °C the agglomerates of size up to 2 μm , consisting of nanosheets of size up to 200 nm and thickness of about 2–5 nm, are formed (Fig. 2).

According to the X-ray phase analysis (Fig. 3), the main peaks of the AlN/Al oxidation products correspond to crystalline boehmite. Both the shift of the reflection in the (020) plane toward smaller angles and the broadening of the peaks indicate the absence of long-range order in the arrangement of atoms and the amorphous structure, which is characteristic for pseudo-boehmite.

Fig. 3 XRD pattern of the agglomerates of AlOOH crumpled nanosheets

Nitrogen N_2 adsorption-desorption isotherms obtained for AlOOH nanosheet agglomerates have a hysteresis loop in the region of capillary condensation at a relative pressure of $P/P_0 \geq 0.4$. Such isotherms are typical for mesoporous materials with slit-shaped pores. The maximum of pore size distribution, determined by the method of Barrett, Joyner and Halenda, is within the region of 4 nm. The specific surface area, determined by Brunauer-Emmett-Teller method was $286 \text{ m}^2/\text{g}$. The slit-like structure of the mesopores and the large specific surface area of the AlOOH nanosheets agglomerates provide them with high adsorption activity (Fig. 4).

Figure 5 shows synthesized nanostructures zeta-potential dependency on pH level of the medium. The nanostructures have a large positive zeta-potential of about 30 mV at pH ~ 6.5 –8.0 and 37°C . The pH of the zero charge point pH_{IEP} of AlOOH nanostructures was estimated as 9.53.

The above results suggest nonequilibrium morphology and structural state of AlOOH nanosheets agglomerates. The crumpled structure causes local deformations and multiple micro-stresses, which contribute to the formation of a larger number of surficial active centers due to surface defect zones. It should be noted that the resulting folded crumpled nanostructures due to the presence of a large number of (active) hydroxyl groups per unit surface area can acquire a significant pH-dependent electric charge. Such aluminum nanomaterial is an amphoteric compound, which in the case of an acidic and even neutral medium relatively easily releases the OH-groups into the solution. Dissociation of OH^- groups is facilitated in the areas of defects of the nanomaterial, similarly to the case of hydroxyl groups deprotonation, observed in cationic clays [39]. Therefore, it can be assumed that AlOOH nanosheets with a

Fig. 4 The N_2 adsorption/desorption isotherms and pore size distributions of AlOOH crumpled nanosheets

Fig. 5 Dependency of zeta potential of ALOOH crumpled nanosheets on pH of the medium at 37 °C

disordered defective surface structure will have a larger positive charge in comparison with regular aluminum oxyhydroxide.

3 Anticancer Activity of Radially Assembled Al Hydroxide Crumpled Nanosheets

Applied to tumor microenvironment, interaction of the positively charged crumpled nanosheets with extracellular anionic species can lead to disturbance of extracellular ion concentration, and thus result in anti-cancer activity. To investigate the anticancer activity of the agglomerates of radially assembled crumpled ALOOH nanosheets, termed Aloohene (“Aloohene”) referring to its chemical formula ALOOH, we have therefore performed a series of *in vitro* and *in vivo* studies using different cancerous cell lines and a mouse melanoma tumor model, respectively.

3.1 Effect of Aloohene on Tumor Cells Viability and Proliferation *in Vitro*

First, we have investigated the effect of the synthesized nanomaterial on the viability of three human cancer cell lines (MCF-7, UM-SCC-14C and Hela) cells. After 24 h incubation of Aloohene, a significant decrease in tumor cell viability was measured for all tested cells compared to the non-treated controls (Fig. 6a). Furthermore, a 30–37% decrease in proliferation of the tested cells was detected using the BrdU (5-bromo-2'-deoxyuridine) assay (Fig. 6b). Taken together, these data indicate that Aloohene affects tumor cells viability, most likely through inhibition of their proliferation.

Fig. 6 Analysis of cellular toxicity of Aloohene. **a** FACS analysis of Annexin V and PI staining of HeLa, MCF-7 and UM-SCC-14C cells with or without co-culture with 5 mg/ml of Aloohene at 37 °C for 24 h. Fluorescence intensity was measured by flow cytometry and data were analyzed by the Cell Quest software. **b** Proliferation of HeLa, UM-SCC-14C and MCF-7 cells, as measured by BrDU assay. Cell cultures were incubated with Aloohene (5 mg/ml) at 37 °C for 24 h. Fluorescence intensity was measured 48 h after BrDU labeling at excitation and emission wavelengths of 370 nm and 470 nm, respectively. Results are means of 3 independent experiments. *** $p < 0.001$. Reproduced with permission from ACS [38]

3.2 Evaluation of Antitumor Activity of Aloohene in Mouse Model of Cancer

We next investigated whether the in vitro cancer cell growth inhibition effect of Aloohene would translate into antitumor efficacy in vivo in a melanoma mouse cancer model based on intradermal administration of 5×10^4 B16F10 cells into the C56BL/6J mice. When the tumor volume reached 70 mm³, mice were treated with Aloohene, doxorubicin and a combination of both reagents. Aloohene was administered at a dose of 10 mg/ml weekly via two intratumoral injections and doxorubicin was injected intraperitoneally at a single dose of 10 mg/kg. The horizontal and vertical tumor diameters were measured by a digital calliper every second day until the end of treatment and volume was calculated using the formula $V = \frac{\pi}{6}ab^2$ where a and b are the longer and shorter diameter of the tumour, respectively. After two weeks of treatment, Aloohene treatment resulted in a significant decrease of tumor growth as compared to the vehicle treated control animals (Fig. 7). A combination therapy with doxorubicin, the standard-of-care anticancer drug, and Aloohene demonstrated a much stronger suppressive effect on tumor growth, also surpassing the anti-tumor effect of doxorubicin alone (Fig. 7).

To address the mechanism of tumor growth inhibition by Aloohene, we have measured the markers of tumor cells proliferation, cell death and vascularization. Notably, a significant decrease in the proliferation rate for all treatment regimens as compared to the control group was measured by immunohistochemical (IHC)

Fig. 7 Antitumor effect of Aloohene in vivo in a mouse melanoma model. **a** Effect of treatment with Aloohene alone and in combination with doxorubicin. Mice were treated with 10 mg/ml of Aloohene, 10 mg/kg of doxorubicin and their combination, and tumor volumes were measured twice a week. Data are presented as mean tumor volume $\pm$ standard errors of mean ($n = 10$ per treatment group). The statistical significance of differences between the groups was assessed by Student's *t*-test. *** $p < 0.001$ compared with the control group. Reproduced with permission from ACS [38]

staining for the proliferation marker Ki67 (Fig. 8), with the most profound effect detected after the combinatorial treatment of doxorubicin and Aloohene.

Further, the level of tumor necrosis, which could be indicative of treatment effect, was assessed by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labelling) staining. Large areas of dead cells were detected by this method in tumors treated with Aloohene, doxorubicin and their combination unlike in the control group ($p < 0.05$) (Fig. 9).

No difference in distribution of the endothelial cell marker CD31 was detected in the analyzed tumor sections, suggesting that tested treatments had no effect on tumor vascularization (Fig. 10). Taken together, these results demonstrate a significant in vivo antitumor effect of Aloohene and its potential to evolve into a novel strategy of cancer treatment, possibly in combination with established chemotherapy drugs, such as doxorubicin.

4 Summary

Collectively, our data demonstrates that AIOOH nanosheets with a disordered defective surface structure that have a large positive charge are capable of disturbing tumor-microenvironment extracellular ion balance, therefore representing a novel class of inorganic materials exhibiting strong antitumor effect. Here we report that Al hydroxide nanostructures in the form of agglomerates of crumpled and radially assembled nanosheets (Aloohene) trigger cancer cell death in vitro and inhibit

Fig. 8 Effect of Aloohene on tumor proliferation in vivo in mouse melanoma model. **a** Cell proliferation in primary melanomas determined by immunodetection of Ki67 in non-treated mice and mice treated with doxorubicin, Aloohene or their combination. Ki67-positive cells calculated as percentage of total cells. **b** Corresponding illustrative images of Ki67 staining in tumors of all tested groups. *** $p < 0.001$ compared with the control group. Reproduced with permission from ACS [38]

Fig. 9 Effect of Aloohene on tumor necrosis in vivo in mouse melanoma model. **a** The percentage of areas of dead cells was determined on high-power fields of primary melanomas of non-treated mice and mice treated with doxorubicin, Aloohene or their combination, based on immunohistochemical detection by terminal dUTP nick-end labeling staining (brown areas). The percentage of proliferative, necrotic and apoptotic cells are presented as means and standard errors, $n = 10$. **b** Representative images are shown for control mice and mice treated with Aloohene, doxorubicin, and their combination. The statistical significance of differences between the groups was assessed by Student's t -test. *** $p < 0.001$ compared with the control group. Reproduced with permission from ACS [38]

Fig. 10 Vascularization of skin melanoma tumors after treatment with Aloohene, doxorubicin or their combination. Representative images of immunofluorescence staining of the endothelial cell specific marker CD31 (red staining) in cryopreserved tumor sections. The scale bar corresponds to 200 μm . Reproduced with permission from ACS [38]

tumor growth *in vivo*. The disturbing effect of Aloohene on ion balance in the tumor-microenvironment is supported by our direct molecular dynamics simulation [38]. Furthermore, our results show that Aloohene nanostructures can potentiate the anticancer action of the cytotoxic agent doxorubicin and thus could improve the efficacy of state-of-the-art chemotherapy when used in combination. The findings of the present research highlight the important role of Aloohene, a novel class of tumor-microenvironment dysregulating nanomaterial based on AlOOH nanosheets agglomerates, in the development of more effective anticancer strategies.

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Author Contributions AL investigated the patterns of synthesis and the physicochemical characteristics of crumpled aluminum hydroxide nanostructures. G. M. and O. V. evaluated the effects of Aloohene on tumor cell growth inhibition *in vitro* and *in vivo*.

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