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Integration of anticancer drug ruboxyl into the membrane of fullerene-based vesicle enhances its therapeutic performance

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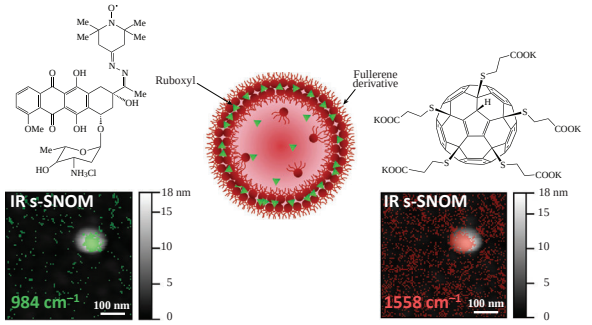
The supramolecular complexation of anthracycline antibiotic ruboxyl with water-soluble fullerene derivative was visualized by scattering scanning near-field optical microscopy, and the effects of this complexation on the antileukemic activity *in vivo* were evaluated. Treatment of mice with P388 lymphocytic leukemia with ruboxyl resulted in 13% of cured animals, while similar treatment with ruboxyl–fullerene derivative combination caused 63% curing.



Keywords: water-soluble fullerene derivative, ruboxyl, leukemia, vesicles, IR s-SNOM.

According to the American Cancer Society, approximately 1.5% of people will be diagnosed with leukemia at some point during their lifetime, based on 2017–2019 data. Currently, combination therapy is considered to be promising for the treatment of leukemia and other oncological diseases. A combination of antioxidants and chemotherapeutic agents demonstrated promising synergistic effects. According to a review[1] that described the effects of combined antioxidant/chemotherapy treatment in 19 randomized clinical trials, 17 of the 19 trials showed the advantages of combination therapy with antioxidants. The effects of this approach should be actively investigated so as it can become the new standard treatment strategy for leukemia in the near future.

Due to the large amount of non-saturated double bonds, fullerene-based compounds are known as highly promising antioxidants.[2] Remarkable results were obtained for both covalent and non-covalent conjugates of fullerenes and their derivatives with anticancer drugs. For example, covalent conjugate of fullerene with doxorubicin (DOX) demonstrates prolonged antitumor activity and a more pronounced antiproliferative effect as compared to non-modified DOX when incubated with MCF-7 cancer cells.[3] Recently, it was reported[4] that covalent conjugation of modified fullerenes with doxorubicin also increased its cytotoxicity against tumor cells. Fullerenol–doxorubicin conjugate suppressed the proliferation of cancer cells *in vitro* by blocking the cell cycle in G2/M phases, leading to apoptosis. In the *in vivo* mice tumor model, fullerenol–doxorubicin conjugate showed antitumor activity comparable to that of doxorubicin, while it lacked the systemic toxicity associated with a single cytostatic agent.[5] A strategy based on simultaneous treatment with fullerenes or their derivatives and anticancer drugs seems to be promising as well. For example, the combination of fullerene with DOX showed more pronounced cytotoxicity in the MCF-7 cancer cell line than pure DOX.[6] The mechanism of antitumor activity of anthracycline drugs such as DOX and its analogues is based on a tight binding of these compounds to DNA. Additional cytotoxicity is caused by the generation of reactive oxygen species, which makes anthracycline antibiotics highly cardiotoxic.[7]

Ruboxyl was developed in the USSR in the 1980s to reduce the oxidative side effects of doxorubicin[8] by its covalent conjugation with paramagnetic TEMPO-type nitroxyl radical (4-hydrazono-2,2,6,6-tetramethylpiperidine-1-oxyl). The nitroxyl moiety of ruboxyl serves as a radical trap. Experiments confirmed a reduced toxicity of ruboxyl as compared to DOX.[8,9] Treatment with ruboxyl extends the lifespan of animals inoculated with experimental leukemia P-388 more efficiently as compared with the effects of rubomycin and rubidazone, and does so in the case of leukemia L-1210. Treatment of Shvets's erythromyelosis with ruboxyl resulted in the inhibition of the tumor growth by 80% as compared to the control, while rubomycin had no effect under similar conditions.[9]

Here we demonstrated that integration of anticancer drug ruboxyl into the membrane of fullerene-based vesicle allowed one to reduce the toxicity of the cytostatic and, in some cases, to increase its therapeutic efficiency *in vivo*. Complexation of the

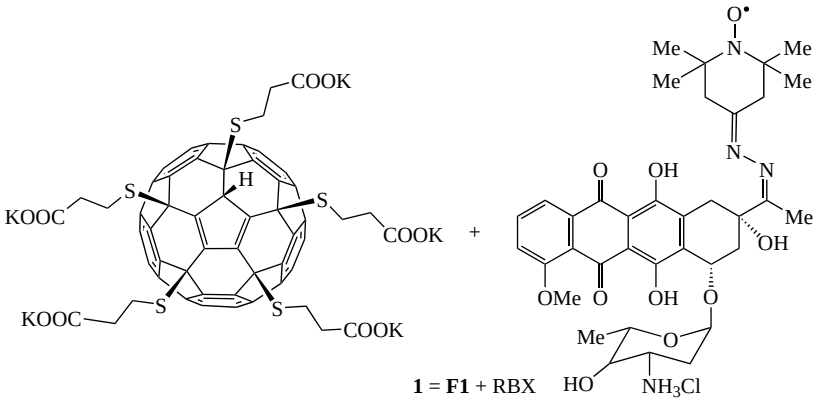


Figure 1 Molecular structures of the water-soluble fullerene derivative **F1** and ruboxyl.

fullerene derivative with antitumor drug was for the first time observed using infrared scattering scanning near-field optical microscopy (IR s-SNOM).

Water-soluble fullerene derivative **F1** (Figure 1) was synthesized and characterized as reported previously[10] from hexachlorofullerene $C_{60}Cl_6$ and 3-mercaptopropanoic acid in the presence of *N*,*N*-diisopropylethylamine (see Online Supplementary Materials, Figure S1). Molecular composition and purity of compound **F1** were additionally proved by matrix-assister laser desorption–ionization (MALDI) mass spectrometry (Figure S2) and gel permeation chromatography (GPC). GPC profile (Figure S3) revealed one peak with retention time of 21.0 min, which proved high purity of the synthesized water-soluble fullerene derivative **F1**.

Amphiphilic fullerene C_{60} derivatives comprising several hydrophilic solubilizing addends on one side of the fullerene cage are known to form various supramolecular structures such as micelles, bi- and multilayer vesicles, and clusters of micelles and vesicles. These vesicles are formed in aqueous solutions due to hydrophobic–hydrophilic interactions, and this process is highly dynamic and can only be controlled by changing the chemical structure of the attached addends,[11] making it impossible to separate the fraction with a specific type and size of clusters.

Non-covalent complex **1** was obtained by joint freeze-drying of aqueous solutions of fullerene derivative **F1** and ruboxyl (see Figure 1). The aggregation behavior of fullerene derivative **F1** and complex **1** was investigated by dynamic light scattering (DLS).[12] The obtained size distribution profiles for both **F1** and non-covalent conjugate **1** are given in Figure 2.

Fullerene derivative **F1** has an extremely narrow particle size distribution. Particles with an average hydrodynamic radius $<R_h>$ of 50.9 ± 12.9 nm (PDI = 0.064) most probably represent bilayer vesicles. Interestingly, aqueous solution of complex **1** contained several types of supramolecular structures. The smallest particles with the hydrodynamic radius $<R_h>$ of 6.0 ± 1.6 nm (PDI = 0.071) most probably represent small micelles, while particles with an average $<R_h>$ of 54.3 ± 23.3 nm (PDI = 0.184) can also be considered bilayer vesicles. The average diameter of the vesicles of complex **1** is about 8 nm higher than the size of the vesicles of pure fullerene derivatives, while the size distribution is more polydispersed. This fact allowed us to suggest that the molecules of antitumor drug ruboxyl were probably incorporated into the membrane of the bilayer vesicle of complex **1** [Figure 2(*c*)].

To additionally support this assumption, we decided for the first time to utilize scattering scanning near-field optical microscopy (IR s-SNOM) for characterization of complex **1**. This is basically the only effective method to study the microstructure of the sample with simultaneous acquisition of

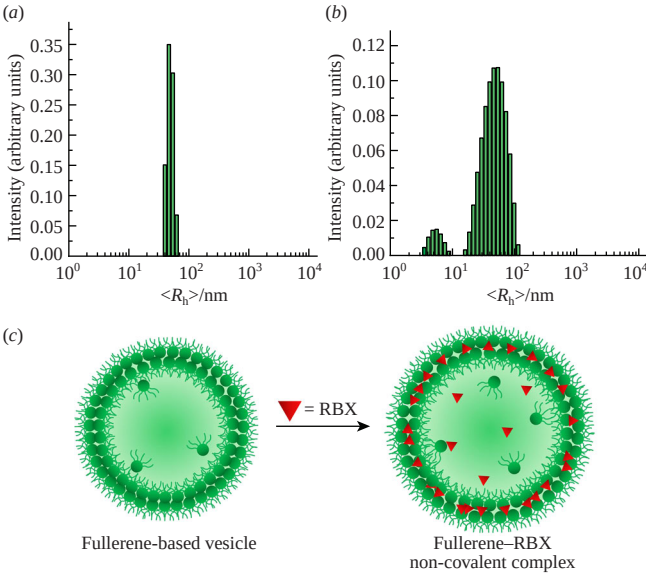


Figure 2 DLS profiles revealing particle size distribution in aqueous solutions of (*a*) the fullerene derivative **F1** and (*b*) non-covalent complex **1**. (*c*) Schematic image of the fullerene-based vesicle incorporating molecules of ruboxyl.

spectral information, which allows one to determine its composition. To determine characteristic vibrational frequencies of fullerene derivative **F1** and ruboxyl, IR spectra of both compounds were recorded (Figure S4). The measurements were performed at the characteristic vibration frequencies of fullerene derivative **F1** and ruboxyl hydrochloride (**F1**: 1558 cm^{-1}, ruboxyl: 984 cm^{-1}) (Figures 3 and S5). The obtained images revealed that vesicles with a radius of about 50–55 nm were present on the substrate, and these vesicles contained both ruboxyl and a fullerene derivative. The data obtained are consistent with the dynamic light scattering results. The SEM results also demonstrated the presence of particles with the size starting from ~50 nm and aggregates with the diameter of >200 nm which were probably formed by fusion of smaller

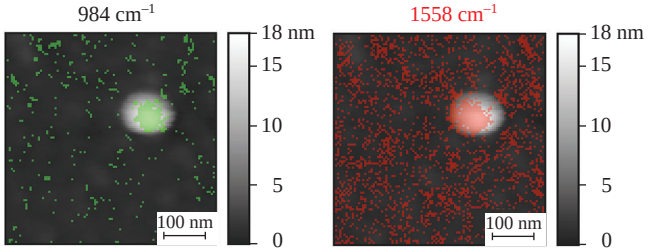


Figure 3 IR s-SNOM image of a vesicle formed in an aqueous solution of complex **1**.

particle during sample preparation (Figure S6). It should also be noted that non-complexated molecules of both fullerene derivative and ruboxyl are also present in the sample, according to s-SNOM data.

Thus, we for the first time demonstrated that IR s-SNOM can be considered an effective method to simultaneous determination of both the size and composition of supramolecular structures formed in the solution of fullerene-based complexes.

The general toxicity of the obtained non-covalent complex **1** was studied on the hybrid mice line BDF_1 under intraperitoneal administration. Toxicological characteristics were calculated for complex **1**: LD_{100} = 331.6 mg kg^{-1}, LD_{84} = 308.7 mg kg^{-1}, LD_{50} = 263.0 ± 10 mg kg^{-1}, LD_{16} = 224.1 mg kg^{-1}, MTD (maximum tolerated dose) = 200 mg kg^{-1} (Figure S7). Notably, a significant decrease in the toxicity was observed as compared to non-modified ruboxyl (LD_{50} = 44 mg kg^{-1}).[9]

Antileukemic activity of both ruboxyl and its complex with fullerene derivative **1** was also investigated in male BDF_1 mice with 22–24 g weight. It should be noted that water-soluble fullerene derivative **F1** did not reveal any antitumor activity in the utilized model. The obtained results are summarized in Tables S1, S2 and Figure 4. Ruboxyl without fullerene derivative was administrated to three groups of animals, each of them comprised 8 animals. The first two groups received 15 mg kg^{-1} of ruboxyl on 1st, 4th, and 7th or 1st, 5th and 9th days. The third group obtained 7 mg kg^{-1} of ruboxyl every day. All three groups demonstrated the increase in the average life span by 91–104%. The first group had one survived animal (13%) on the 60th day after the beginning of observation, while all of the animals in the second and the third groups died. Complex of the ruboxyl with water-soluble fullerene derivatives was also administrated to three groups of animals using the same administration modes. Although the amount of the administrated complex was 50 and 25 mg kg^{-1}, these doses contained the same amount of ruboxyl (15 and 7 mg kg^{-1}) as in the first three groups. Thus, the only difference between these experiments was the presence of the water-soluble fullerene derivative **F1**.

Treatment of the animals with fullerene–ruboxyl complex at a concentration of 50 mg kg^{-1} on the 1st, 4th and 7th days led to an increase in the number of surviving animals up to 63% (5 animals out of 8), while in the second treatment scheme (1st, 5th and 9th day) 25% of animas survived on the 60th day. Additionally, administration of the complex at 1st, 4th, and 7th days allowed us to increase the average life expectancy by 155% (for those animals, who died), while mean survival time was the highest among all six treatment schemes. As compared to the results obtained for pure ruboxyl, experiments with the administration of fullerene–ruboxyl complex (25 mg kg^{-1}) every day for one week led to slightly less promising results. Thus, *in vivo* experiments demonstrated that supramolecular complexation of ruboxyl with water-soluble fullerene derivative could enhance the therapeutic effect of the antitumor drug after the optimization of the administration mode.

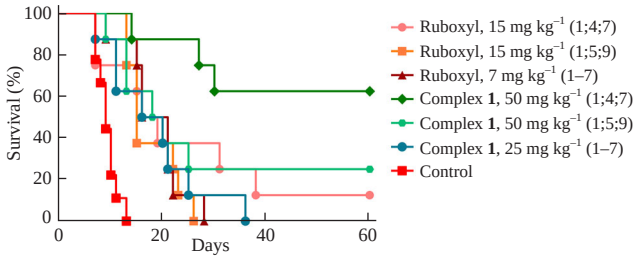


Figure 4 Kaplan–Meier curves demonstrating the survival of mice with P388 leukemia in time.

The reason behind the enhancement of antitumor activity is a subject for further investigation. However, several assumptions can be made from the existing data. Currently, evidence is growing that antioxidants may provide some benefit when combined with certain types of chemotherapy.[1] The ability of the fullerene derivative **F1** to scavenge free radicals *in vivo* due to the presence of a high amount of double bonds in its structure can influence the results of antileukemic treatment. Complexation can also aid in the optimization of drug delivery and affect the distribution in the body.

Thus, integration of the anticancer drug ruboxyl into the membrane of fullerene-based vesicle was demonstrated using dynamic light scattering and scattering scanning near-field optical microscopy. A significant decrease in toxicity *in vivo* was observed for supramolecular complex as compared to non-modified ruboxyl. In mice P388 lymphocytic leukemia model, fullerene–ruboxyl complex demonstrated enhancement in its therapeutic performance as compared to pure ruboxyl.

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Online Supplementary Materials

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2024.02.006.

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