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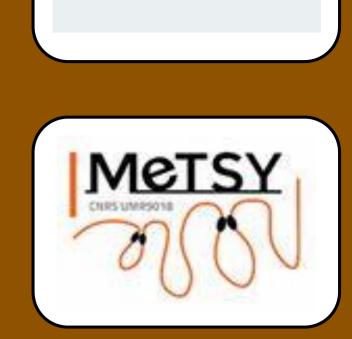


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EMBEDDING VIRAL CAPSIDS INTO AGGREGATED METALLIC NANOPARTICLES IMPROVES ADENOVIRUS ENTRY IN VARIOUS HUMAN CANCER CELLS

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INTRODUCTION

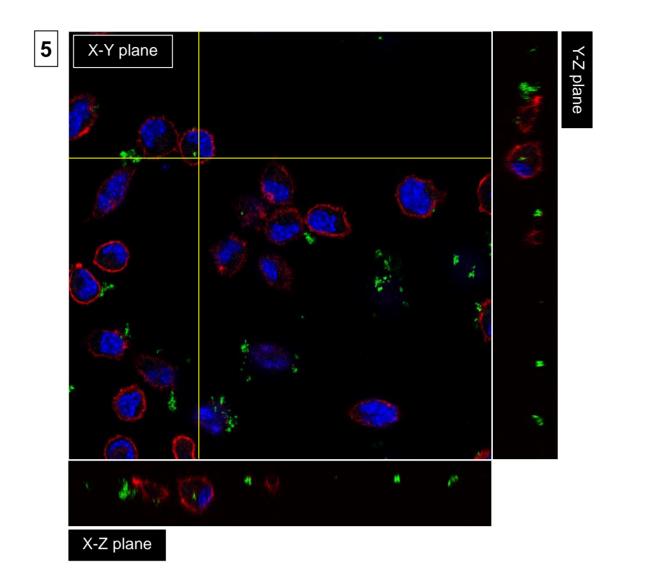
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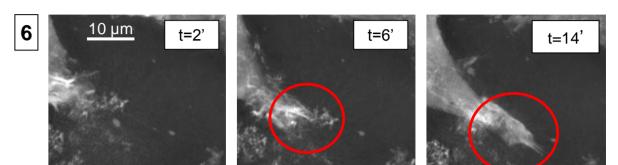
ROUSSY

Background: Delivery of macromolecules or nano-objects by electrotransfer is an attractive procedure in many fields of biotechnology and gradually in therapeutics. When using low amplitude/long lasting electric pulses to facilitate adenovirus delivery into cancer cell lines, we previously showed an enhanced virus penetration, not directly resulting from pulse application nor to membrane modifications, but rather due to viral capsid interactions with aggregated metallic nanoparticles, released from energized electrodes.

RESULTS

Penetration of adenovirus/aluminum aggregates into cells

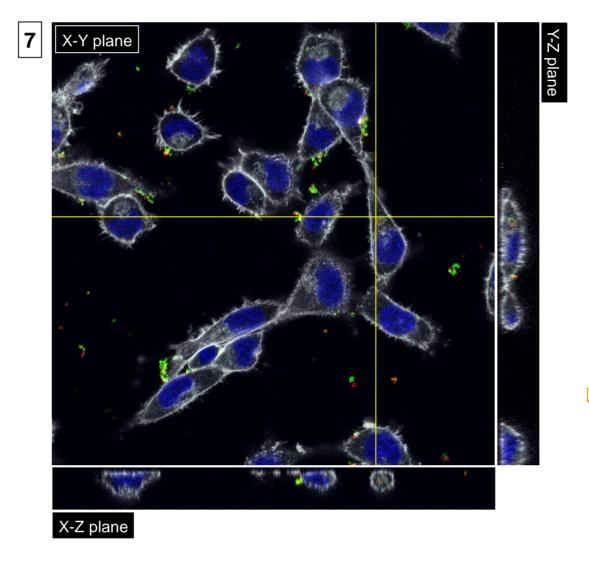


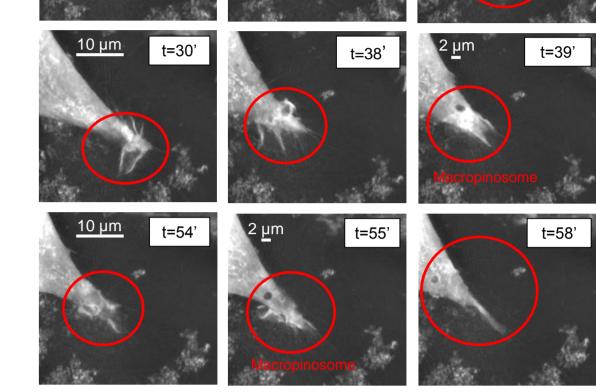


<u>Results</u>: In this work, we have quantified the viral and metallic particles forming these complexes by nanoparticle tracking and Inductively Coupled Plasma-Atomic Emission Spectroscopy, respectively. The intimate structure of these complexes and their cell penetration were investigated by transmission electronic microscopy and confocal microscopy. The new properties conferred on viral capsids by aggregated metallic nanoparticles were further studied. We characterized their contribution to virus stability and to penetration of embedded viral capsids into cell lines derived from several human cancers, including some Head and Neck carcinoma cell lines poorly infected with adenovirus vectors. Using a panel of endocytosis inhibitors, we found that macropinocytosis is the main penetration pathway for the viral capsids associated with the metallic aggregates.

MATERIALS AND METHODS

- Cell culture and infection: L929, LLC (murine cell lines), ACHN (renal cell adenocarcinoma), FaDu and Detroit (H&N carcinomas) were cultivated in DMEM and 10% FCS; CHO and CHO-hCAR (gift of J.M Bergelson) negative and positive control for the presence of CAR (Coxsackie and Adenovirus Receptor) were cultivated in F12 medium and 10% FCS. A purified recombinant adenovirus (rAd) expressing the Green Fluorescent Protein under the control of the cytomegalovirus promoter was used to infect cells directly ("free virus") or after or after embedding in aluminum aggregates, at the multiplicity of infection (MOI in viral particle/cell) specified. Cells were held on ice during their mixing with viral solution.
- Electric pulse delivery and treatments: electric pulses were delivered from CliniporatorTM device with low voltage pulses: 10 x 20 ms, 10 Hz, 10 kV/m), in standard 1 mm-wide cuvette with plate aluminum electrodes. S-MEM medium was pulsed without virus to obtain pure aluminum (AI) aggregates. Similarly, S-MEM medium + rAd mixture (infection medium) was pulsed to obtained virus capsid association to AI aggregates as described in Fig. 1/2. Then the content of the cuvettes was submitted to a double centrifugation (300 g at 4°C), the supernatant and the pellet were conserved for the use in other experiments and infection of cells (Fig. 3). Samples of either the pulsed infection medium or the pulsed S-MEM mixed a posteriori with the virus, were contrasted by negative staining method using uranyl acetate dye and treated to be analyzed in TEM (Fig. 4).
- Gene expression analysis: 24 h after rAd-mediated gene transfer, GFP expression efficiency (% of GFP-positive (GFP+) cells) and GFP expression intensity (median of GFP+ cell fluorescence in RLU) were analyzed by FACS for different treatments in duplicates (Fig. 9-11).
- > Specific analysis: Fig. 3 : Quantification of aluminum mass of aggregates was realized on wet centrifuged pellets by





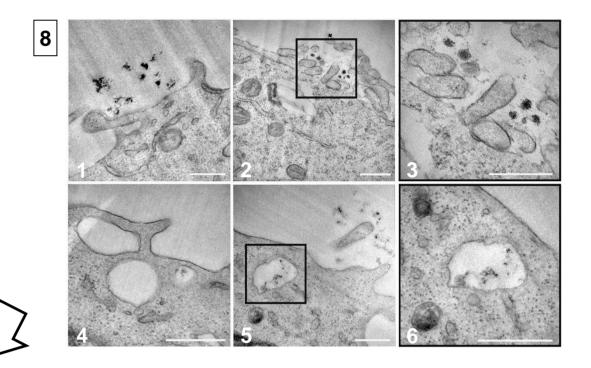


Fig. 8 : Aggregated metallic particles formed without (1/4) or with Ad (2/3/5/6) with characteristic elements of macropinocytosis. 1-3: formation of membrane ruffles and large macropinosomes (Ø > 0.2 µm) seen in 4-6. LLC cells, in absence or presence of Ad (MOI = 2x10⁴ or 6x10⁴ pv/cell in 2/3 and 5/6, respectively), and treated for TEM as indicated after an incubation lasting 10 (2/3), 45 (1/5/6), or 55 min (4) at T=37°C. Scale bar = 0.5 µm.

New properties conferred on viral capsids by aluminum aggregates

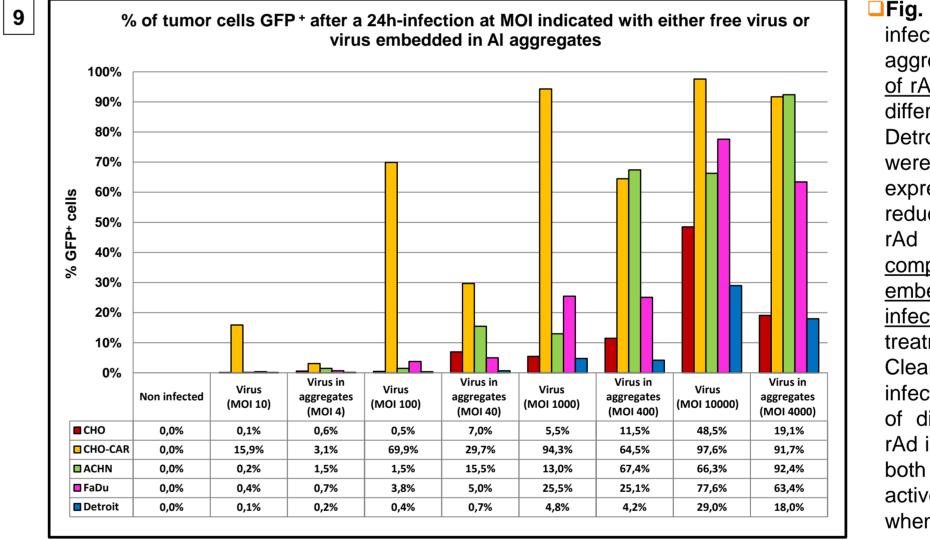


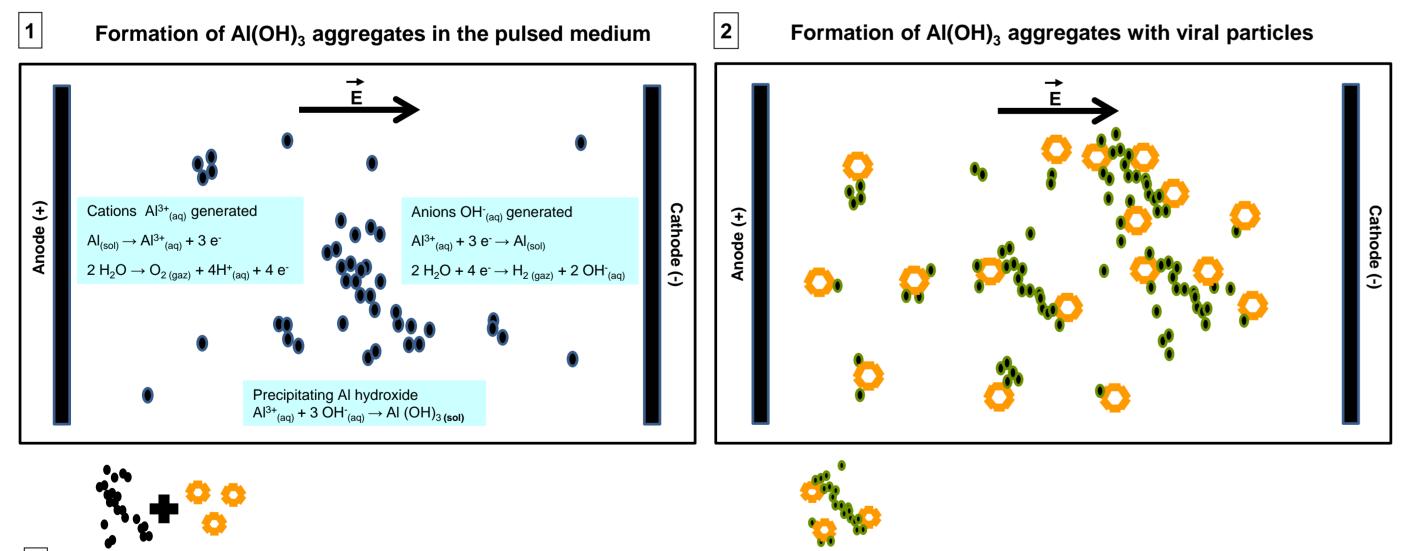
Fig. 9-11 : GFP expression analysis 24h after infection with free rAd or rAd embedded in Al aggregates. Fig. 9 : comparison of efficiency of rAd embedded or not in aggregates to enter different human tumor cells (ACHN, FaDu and Detroit) at different MOI. CHO and CHO-hCAR were cell controls for negative or positive expression of CAR, respectively. A MOI reduction of a factor 2 to 10 was observed for rAd embedded in Al aggregates. Fig. 10 comparison of the thermal stability at 37° of rAd embedded or not in Al aggregates for the infection of L929 cells. Duration of the therma treatment before infection was indicated Clearly, rAd in Al aggregates do not loose its infection capacity with the time. Fig. 11 : effect of different endocytosis inhibitors to prevent rAd infection of FaDu cells. Amiloride and DMA both inhibiting macropinocytosis, were the most active to reduce rAd entry and particularly when embedded in Al aggregates.

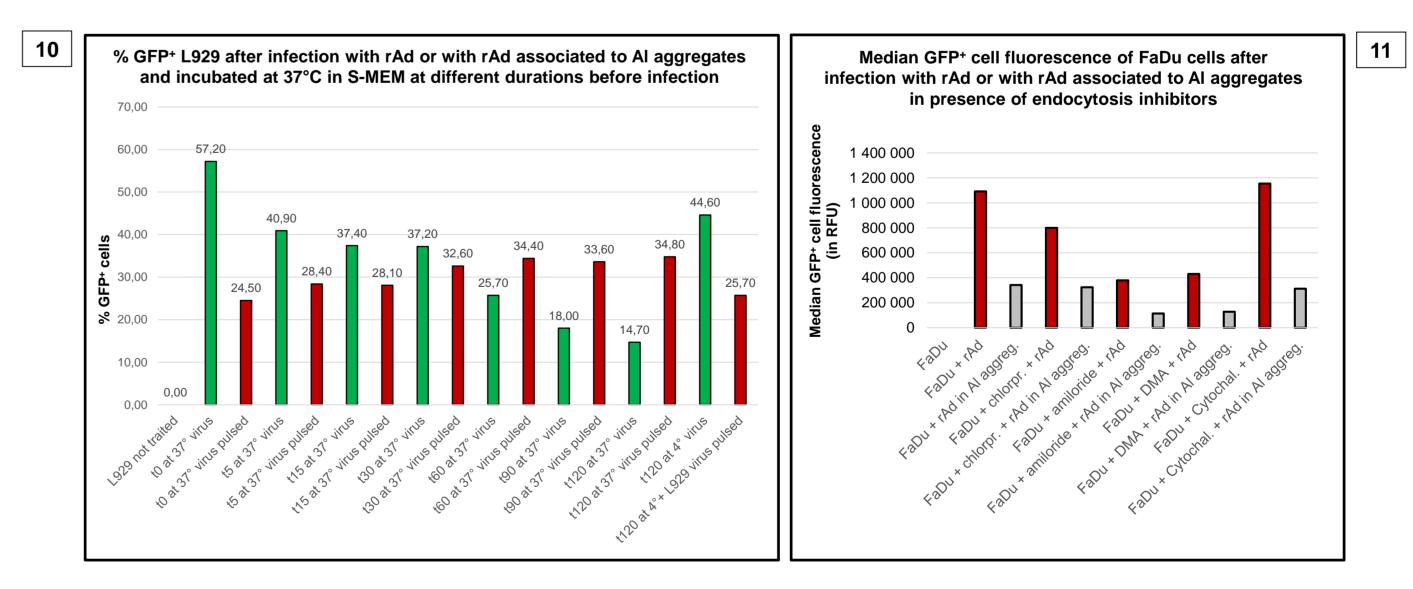
Inductively Coupled Plasma–Atomic Emission Spectroscopy (ICP-AES) analysis with a iCAP 6300 radial ICP emission spectrometer (ThermoFischer Scientific). **Fig. 5-8** : Different experiments analyzing aluminum aggregate (associated or not to adenovirus particles) entry into cells (L929 or LLC cells) and showing evidences of macropinocytosis. **Fig. 5/7** : exemples of confocal microscopy experiments on L929 cells exposed 15 min or 1 h to either aluminum aggregates alone or to Ad associated to aggregates, respectively, and treated after fixation with different dyes. Orthogonal view from the 3 different planes (x/y, x/z or y/z) of the confocal microscope images were used to analyze the particle uptake. Cell nuclei were stained with Hoechst (blue), cell perimeter (polymerized actin F) with phalloïdine Alexa Fluor 647 (red or white, in Fig. 5 and 7, respectively), dextran 10K Oregon Green 488 (green) was added with aggregates to stain them, and in Fig. 7, adenovirus particles were stained with Star Orange (ex:589/em:616) before their association to aggregates. Orthogonal yellow lines point to specific Al aggregate and adenovirus particle staining observed inside cells. **Fig. 6** : Thanks to Nanolive Company, we use their 3D Cell Explorer holotomographic platform, based on refractives indexes, to see the entry of Al aggregates into cells without the use of any labels. After adding Al aggregates to the cells, they were incubated 1h at 37°C and several keyframes of part of the same cell were taken at the time indicated, to show each macropinocytosis events observed. **Endocytosis inhibitors:** rAd associated or not to Al aggregates was added to cells previously treated during 1 h with :

Endocytosis inhibitors: rAd associated or not to AI aggregates was added to cells previously treated during 1 h with : sodium chlorpromazine (10 μg/ml), amiloride (500 μM), DMA (dimethyl amiloride) (50 μM), cytochalasine D (2 μg/ml), and after 1 h infection, cells were incubated 24 h with inhibitors to test GFP expression (Fig. 11).

RESULTS

Adenovirus viral capsid embedding and quantification





CONCLUSION AND PERSPECTIVES

Viral capsids embedded in aluminum aggregates enter cells mainly by macropinocytosis as shown here. This process allows the entry of rAd in human tumor cells expressing



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Supernatant => free virus = 90% +/- 5%Pellet => virus associated to $AI(OH)_3$ aggregates = 10% +/- 5%=> $AI(OH)_3$ mass # $32 \mu g$ per $10^9 v p$



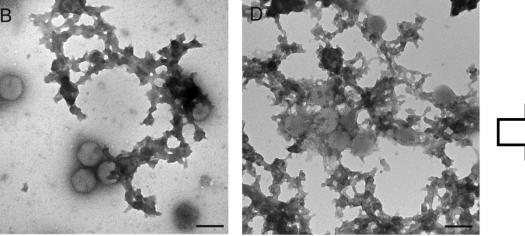


Fig. 3 : Quantification of adenovirus particles: percentages of adenovirus particles associated to aluminum aggregates (present in the pellet after centrifugation) were deduced from the total number of virus particles, either added to the already formed aluminum aggregates (left experiment - Fig1) or added in the cuvette before the aluminium aggregate formation, during applications of electric pulses (right experiment - Fig2), minus virus particles number found in the supernatant after centrifugation. Both concentrations were measured by Videodrop (Myriade Company, France), an optical system based on interferometric light microscopy that allows to rapidly measure the size and concentration of nanoparticles (80-500 nm) in small volumes (5-15 µl), with a concentration range of 10⁸ to 10¹⁰ particles/ml.

Supernatant => free virus = 60% +/- 10%

aggregates = 40% + - 10%

=> Al(OH)₃ mass # 8 µg per 10⁹ vp

Pellet => virus associated to $AI(OH)_3$

Fig. 4 : <u>TEM study</u> showed that more « free » (not associated to metallic aggregates) virions were observed when the virus was added after the formation of metallic particles in the pulsed medium (left panel), compared to the case where the virions were already present in the medium during the pulse (right panel). A and C show large views at low magnification (x4400). Detail of the aggregates at high magnification (x79000) are seen in B and D. Scale bar = 2 µm (A,C); 100 nm (B,D).

low-levels of CAR receptor, since the virus is made independent of its presence to enter cells. In addition, it seems that aluminum aggregates give a thermal stability to viral capsids, probably because the capsid no more needs the fibers that anchor it to the membrane receptor of host cell and which are the most fragile parts of the virus. In conclusion, the properties conferred by aluminum aggregates could be useful to reduce the virus doses required for tumor treatments with rAd, particularly when tumor cells express low levels of CAR protein.

These results must be confirmed in 3-D cultures of human tumors including H&N carcinomas. We will also test if the modification of the cell entry process might modulate the quality and intensity of the immune response induced against the viral vector.

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