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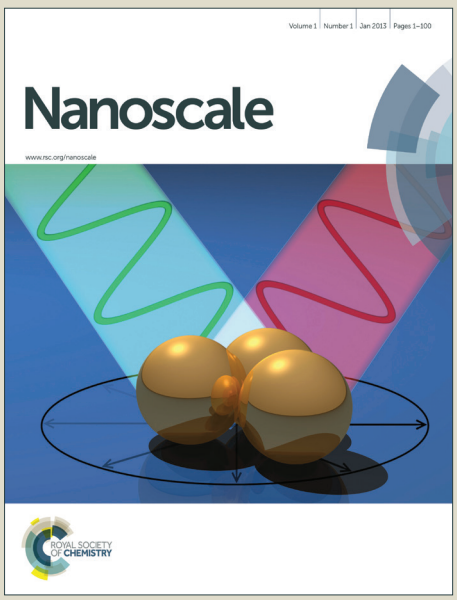

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Cytoprotective Nanoparticles By Conjugation of a Polyhis Tagged Annexin V to a Nanoparticle Drug

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We synthesized a cytoprotective magnetic nanoparticle by reacting a maleimide functionalized Feraheme (FH) with a disulfide linked dimer of a polyhis tagged annexin V. Following reductive cleavage of disulfide, the resulting annexin-nanoparticle (diameter = 28.0±2.0 nm by laser light scattering, 7.6 annexin's/nanoparticle) was cytoprotective to cells subjected to plasma membrane disrupting chemotherapeutic or mechanical stresses, and significantly more protective than the starting annexin V. Annexin-Nanoparticles provide an approach to the design of nanomaterials which antagonize the plasma membrane permeability characteristic of necrosis and which may have applications as cytoprotective agents.

Cytoprotective strategies are sought in a variety of contexts such as reducing gastrointestinal tract irritation,[1] protecting normal cells from chemotoxic cancer agents,[2] and reducing the effects of ischemia.[3, 4] Cytoprotective nanomaterials include metal-polyphenol nanoshells that protect yeast cells[5] and autophagy enhancing fullerenes which protect against toxic effects of the beta-amyloid peptide.[6] Recently, we noted that the annexin V-magnetic nanoparticle known as anx-CLIO(Cy5.5), which we developed in 2004,[7] and which we used to image phosphatidylserine (PS) expression by MRI,[8, 9] reduced ischemia/reperfusion injury in the mouse heart.[10]

With the goal of enabling further studies on the protective effects of annexin V-nanomaterials, we present here a synthesis of annexin V-nanoparticles where a polyhis tagged annexin V (anx) is conjugated to a Feraheme (FH) nanoparticle (NP). Annexin V is a mediator of PS induced coagulation and inflammatory reactions.[11] The efficient targeting of Annexin to PS on apoptotic cell membrane is key to cytoprotection of stressed cells.[12] A dimer of annexin V, diannexin, blocks blood mediated coagulation reactions[13] and inflammatory infiltration of pancreatic islet graft.[14] Diannexin is in clinical trials for protection against reperfusion injury in the kidney transplantation.[15]

Here we show that annexin V-nanoparticles denoted "anx-FH" have a cytoprotective effect against chemotherapeutic or mechanical stresses in multiple cell lines. By using FH NPs, that are approved for treating iron anemia in the US and Europe, anx-FH uses a widely available, nontoxic NP. By using a polyhis tagged, anx-FH uses an anx that is overexpressed in bacteria and easily purified. The anx-FH NP thus is amendable to large-scale synthesis for more extensive cell-based studies of NP cytoprotective activity, or in disease model animals far larger than the mouse (e.g. infarcted pig heart model).

Feraheme's (FH, AMAG Pharmaceuticals, Cambridge, MA) carboxyl groups were converted to amines by reaction with ethylene diamine,[16, 17] to yield reactive 15-20 reactive amines per particle (5874 Fe/NP). Aminated FH was then reacted with 10 equivalents per amine of succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC, Molecular Biosciences, Boulder, CO) for 45 minutes at room temperature. Excess SMCC was removed by NAP-5 column (GE Healthcare, Waukesha, WI). Reaction with SMCC was verified by the loss of amines with NHS ester of CyAL5.5[18] and was greater than 95%.

Below we give the materials and methods for each of the four reactions shown in Figure 1.

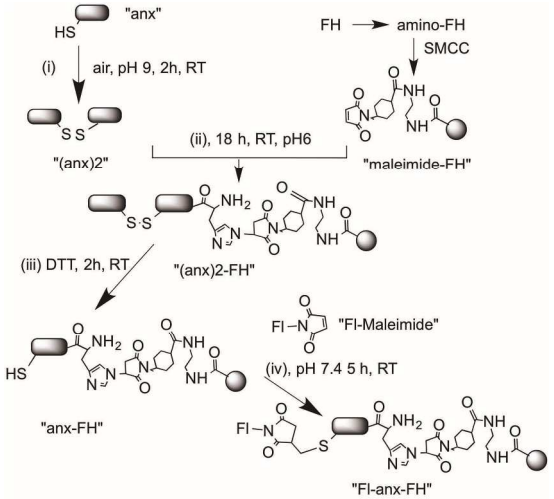

Figure 1. Synthesis of Annexin V-Nanoparticle. Anx was dimerized in reaction (i), reacted with a maleimide-FH NP in reaction (ii), and treated with DTT to cleave the anx dimer, (anx)2 in reaction (iii). To confirm a reactive thiol had been generated, anx-FH was reacted with a fluorescent-maleimide (Fl-maleimide, (iv)). Maleimide-FH is a maleimide-bearing version of the Feraheme (FH) nanoparticle used to treat iron anemia.

Reaction i, (anx)2: The plasmid for annexin V was generously provided by Dr. H. Martin[19, 20] and sent to Blue Sky Biotech, (Worcester, MA) for production in E. coli. Anx was >96% pure by capillary electrophoresis using an Agilent Bioanalyzer. Anx was concentrated with a 10 kDa cutoff ultrafilter, (Amicon Ultra-0.5 ml Centrifugal Filter, Millipore, Billerica, MA) and suspended in PBS, pH 9, 2 mM EDTA. To obtain an disulfide linked anx dimer "(anx)2", a solution of anx (37.5 mg/mL) was oxygenated by vigorous stirring (4°C, 48 hours). (anx)2 was concentrated (50 kDa Amicon Ultra-0.5 ml, Millipore), eliminating traces of anx.

Reaction ii, (anx)2-FH: Conjugation of (anx)2 to maleimide-FH was carried out in PBS, 2.5 mM EDTA, pH 6 for 18-20 hours, room temperature. Purification was by dialysis using a 100 kDa, cellulose ester membrane, (Spectrum Labs, Rancho Dominguez, CA) in PBS at pH 7.4, overnight, 4°C.

Reaction iii, anx-FH: (anx)2-FH was reacted with 5 mM dithiothreitol (DTT) for 2 hours at room temperature, pH 7.4 with purification by dialysis as above.

Reaction iv: anx-FH or (anx)2-FH was reacted with 20 fold excess of HiLyte Fluor 647 C2 Maleimide (Anaspec, Freemont CA) for 5 hours in PBS, pH 7.4, at room temperature. The reaction was monitored by FPLC.

Protein concentration was measured using the DC assay kit (BioRad, Hercules, CA). Iron content was quantified by absorbance at 400 nm using a FH standard (Evolution 300 UV-VIS Spectrophotometer, Thermo Fisher Scientific, Waltham, MA). Nanoparticle amines (amino-FH, or maleimide-FH) were determined by reaction with an NHS-ester of CyAL5.5, its removal on unreacted fluorochrome using a NAP column, using an extinction coefficient of 130,000 mM^{-1} cm^{-1} at 674 nm.[18] A Fast Protein Liquid Chromatography system (FPLC, AKTApurifier, GE Healthcare, Little Chalfont, UK) with a Superdex 200 10/300 GL column (GE Healthcare) was used. Nanoparticle size was measured by dynamic light scattering (DLS) with a Zetasizer (Malvern Instruments, Marlboro, MA). Results were expressed as mean ± 1 SD, n =3.

The bioactivity (apparent affinity) of anx-FH was assessed by its ability to block the binding of a commercial fluorescent annexin from camptothecin (CPT) treated CHO cells.[7] CHO cells were first incubated with CPT (5 μM, 24 hours, 37°C) to induce phosphatidylserine exposure, and then allowed to bind anx-FH in annexin-binding buffer (20 minutes, 37°C and 5% CO2). Cells were washed 3 times with dPBS+1%FBS, incubated with fluorescent annexin (Life Technologies, Carlsbad, CA), (10 minutes, 37°C) and analyzed by FACS in Annexin Binding Buffer (Life Technologies), diluted to 1x with distilled water and 1% fetal bovine serum added.

Anx-FH protection of plasma membrane disruption was assessed with HL-1, A549 and CHO cells. Chemotherapeutic stress was by exposure to 5 μM CPT for 12 hours. To stressed cells ($0.5\text{-}1x10^6$), a putative protective agent was added: anx-FH or FH (0.5 μg Fe/ml) or anx (80 ng), with further incubation for 4 hours. 3 μg/ml propidium iodide (Life Technologies) was added (15 minutes, room temperature) and the percent of cells binding propidium determined by FACS. Mechanical stress was induced in HL-1, A549 and CHO cell lines. Briefly, $0.5\text{-}1x10^6$ healthy cells were stressed by sonication (Bransonic 3510R-MT, Branson Danbury, CT) for 3 cycles of 5-minute sonication and 1-minute rest before the addition of anx-FH or Annexin-Binding buffer. After 2-hour incubation (37°C, 5% CO_2), cell membrane integrity was measured as described above by FACS and PI. Each experimental condition was performed in triplicates and repeated on 3 independent occasions (n=9).

Statistical analysis was with Prizm (Graphpad, La Jolla CA). ANOVA with Tukey post test was used to compare the hydrodynamic size of FH, anx-FH, (anx)2-FH and to compare the PI positivity after treatment with buffer control, anx-FH, annexin alone, FH alone. Unpaired t test was used to compare membrane integrity of cells exposed to anx-FH particle or buffer only. $p < 0.05$ was used as cutoff for statistical significance.

Anx-FH was synthesized (Figure 1) by reacting a maleimide-functionalized Feraheme (FH) nanoparticle (NP) with a disulfide linked dimer of polyhis tagged, wild type human annexin V ((anx)2, (reaction ii), which, after reduction with dithiothreitol (DTT, reaction iii), yielded anx-FH. Finally the anx-FH was reacted with a maleimide fluorochrome (reaction iv). Support for the reactions shown in Figure 1 is provided in Figure 2, and the advantages of the synthesis discussed below.

Reaction i, anx dimerization: FPLC chromatograms of the monomer, anx, and dimer, (anx)2, are shown in Figures 2a and 2b, respectively. As expected, annexin monomer and dimer had the globular equivalent molecular weights of 36 kDa and 72 kDa by FPLC using globular protein standards.[21, 22] A small amount of anx aggregate formed during dimerization.

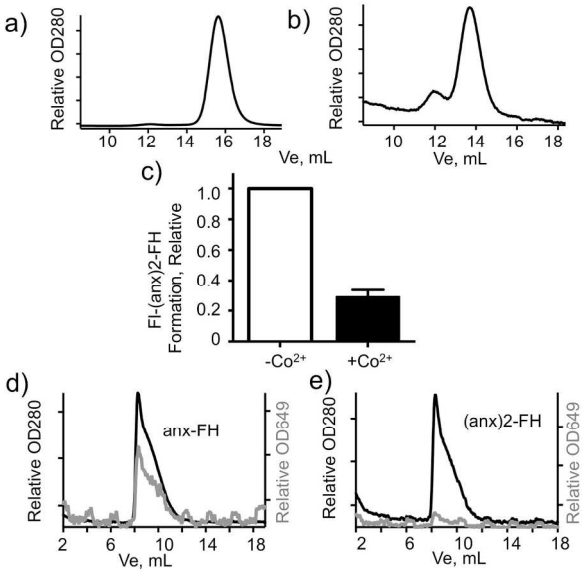

Figure 2: Analysis of reactions used in the synthesis of anx-FH. a) FPLC chromatograms of the annexin V monomer, anx (a) and the annexin dimer, (anx)2 (b), are shown. c) Effect of cobalt ion (Co2+) on the reaction between (anx)2 and the maleimide-FH nanoparticle. Co2+ blocks this reaction. Reaction of Fl-maleimide with anx-FH is shown in (d) or lack of reaction is shown with (anx)2-FH in (e). Anx-FH but not (anx)2-FH reacts with Fl-maleimide, indicating the former has an unprotected free thiol.

Reaction ii, reaction of (anx)2 with Maleimide-FH: The carboxyl groups of Feraheme (FH) were converted to amines 15 and reacted with SMCC (Succinimidyl-4-(N-Maleimidomethyl) Cyclohexane-1-Carboxylate), to yield "Maleimide-FH." The reaction between maleimide functionalized NP's and a histidine on anx is shown (Figure 1) and because the pH of 6 used for the conjugation is close to the pKa of a imidazole (pKa ~ 6.5) but well below the pKa of primary nitrogens (pKa ~ 12) and because of the ability of Co2+ bind to histidine[23] and block the reaction between (anx)2 and maleimide-FH (Figure 2c). In addition the reactions of an imidazole nitrogen with maleimides has been described.[24]

Reaction iii, reduction of (anx)2-FH to yield anx-FH: DTT reduced the numbers of anx's per NP from 12.6 to 7.6 and reduced NP size by light scattering from 29.6±0.8 nm to 28.0±2.0 nm ($p < 0.05$, Figure 3a).

Reaction iv of anx-FH with a maleimide-Fluorochrome: Our scheme (Figure 1) requires (anx)2-FH should lack, while the anx-FH

NP should have, a thiol. We therefore reacted both NP's with HiLyte Fluor 647 C2 Maleimide ("Fl-maleimide"), obtaining a fluorescent NP with anx-FH (Figure 2d) but not with (anx)2-FH (Figure 2e). Reaction iv yields a now fluorescent and magnetic anx-NP and serves to prove the availability of a thiol on anx.

The size of NPs was determined by laser light scattering as shown in Figure 3a. (anx)2-FH at 29.6±0.8 nm (12.6 anx/NP) was significantly larger than anx-FH at 28.0±2.0 (7.6 anx/NP) and which in turn was larger than FH at 24.0±0.7 nm. Thus NP diameter correlated with the number of anx attached per NP.

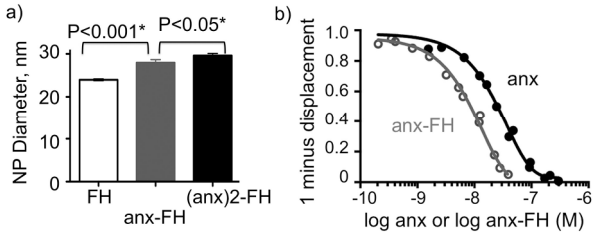

Figure 3: Size and bioactivity of anx-FH. a) Sizes of FH, (anx)2-FH and anx-FH by light scattering are show. Values are given in Table 1. FH was smaller than anx-FH. Anx-FH was smaller than (anx)2-FH. b) Bioactivity of the anx-FH to apoptotic cells. Activity was measured as the displacement of a fluorescent-anx from apoptotic CHO cells by FACS. EC50's are given in Table 1.

The affinities for the anx-FH NP and anx were determined by their ability to displace a fluorescent-annexin from PS expressing cells by FACS (Figure 3b). Anx had an apparent affinity of 29.7 nM while the apparent affinity for the anx-FH NP was 19.1 nM, with 95% confidence intervals given in Table 1.

Table 1. Properties of Annexin-Nanoparticles Summary

Material	Size, nm*	Anx/NP	EC50
Anx-FH	28.0±2.0 nm (Fig. 3a)	7.6	19.1 nM (6.8-54 nM) (Fig. 3b)
(Anx)2-FH	29.6±0.8 nm (Fig. 3a)	12.6	
Anx-CLIO(Cy5.5)	50 nm**	3.5**	
FH	24.0±0.7 nm (Fig. 3a)	Not appl.	
Anx	36 kDa	Not appl.	29.7 nM (25.5-35.5 nM) (Fig. 3b)

* Laser light scattering, ** [7]

We next investigated the cytoprotective effects of anx-FH as shown in Figure 4. Cells were subjected to two very different types of plasma membrane disrupting stresses: a mechanical stress (sonication) and a chemotherapeutic stress (camptothecin, CPT). CPT produces apoptosis, necrosis and annexin V binding over several hours in various cell lines, presumably reflecting activation of specific cell death pathways.[25] The mechanical stress of a brief sonication provided a far faster and far different way of generating plasma membrane rupture (five minutes of sonication, 3 times).

Stressing cells by either method increased the membrane compromised, propidium binding fraction determined by FACS, shown for chemotherapeutic stress in Figure 4a. When CHO cells were stressed by CPT (Figure 4b), anx-FH had significantly more protective effect than anx, $p<0.01$. Anx-FH's protective effects were then examined with three cell lines with chemotherapeutic or mechanical stresses (Figures 4c, 4d). Our anx-FH was highly protective against plasma membrane disruption, whether disruption was caused by a prolonged exposure to camptothecin (16 hours) or a short duration mechanic stress. Anx-FH is directly cytoprotective, or protective against plasma membrane rupture with cultured cells, in the absence of circulating proteins and cells that participate in the thrombotic or inflammatory reactions which diannexin has been noted to decrease, see above.

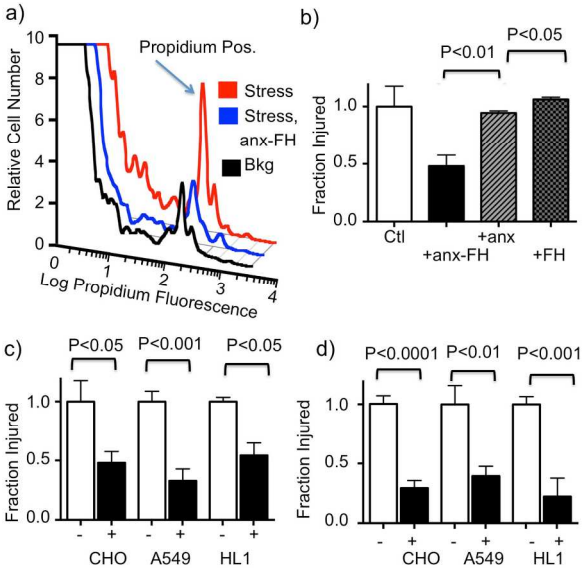

Figure 4: Cytoprotective effect of anx-FH against membrane rupture by chemotherapeutic stress or mechanical stress. Propidium enters cells with a disrupted plasma membrane but is excluded when membrane is intact. a) Single channel FACS of propidium stained CHO cells subjected to mechanical stress (sonication) with protection by anx-FH. Cell cultures have a small percentage background of propidium positive cells (black). Mechanical stress from sonication increased propidium-binding fraction (red). Anx-FH reduced propidium-binding cells (blue). b) Anx-FH and anx protection from camptothecin induced chemotherapeutic stress. The fraction of injured cells is calculated from the three PI positive fractions shown in (a). Fraction Injured is: (+Stress with anx-FH – No Stress Bkg) / (+Stress with no anx-FH – No Stress Bkg). Fraction Injured is 1.0 without protective agents. Anx-FH is more protective than anx. c) Anx-FH protection from chemotherapeutic stress with three cell lines. d) Anx-FH protection from mechanical stress with three cell lines. Anx-FH is more protective than anx and is protective with two very different types of insults producing plasma membrane rupture.

The synthesis and design of the anx-FH NP used in our cytoprotective studies has a number of notable features.

We employed the Feraheme (FH) nanoparticle (NP) because FH is approved for treating iron anemia in the US and Europe and widely available. FH is also an extremely heat stable NP, evident by its terminal sterilization at 121°C. In addition, FH features a coating of carboxymethyl dextran that provides carboxyl groups that can be readily converted to amines.[16, 17] FH has a core of superparamagnetic iron oxide and can be used as an MR contrast agent.[26, 27]

We employed a polyhis tagged annexin V,[19, 20] which is expressed at high levels in bacteria and is readily isolated in large quantity and high purity. We used SMCC to obtain the maleimide-FH NP's which could be reacted with (anx)2, a disulfide linked annexin dimer with two polyhis purification tags. The maleimide ring of SMCC is highly stable and suitable for the relatively slow reaction of maleimide-FH with (anx)2.[28] This strategy avoids the use of N-hydroxysuccinimide esters like the N-succinimidyl S-thioacetate (SATA), used in the synthesis of anx-CLIO(Cy5.5). Annexin V looses bioactivity with the modification of one amine group per mole by N-Hydroxysuccinimide esters like SATA[7] or fluorochromes,[29] so the reaction with SATA must be carefully controlled to obtain reasonable yields of anx-NPs.

We reacted the maleimide-FH with the disulfide linked annexin dimer "(anx)2" (reactions ii and iii) because it yielded a NP with a high level of 7.6 anx's/NP for anx-FH, with size of only 28 nm (Table 1). This equates to an average density of one annexin molecule per 324 nm^2 of the spherical FH NP surface. The high local density of anx is unachievable without FH. This simple two-component design (Anx and FH), could be further modified with maleimides (reaction iv).

Our cytoprotective assay determines the ability of a compound to decrease the number of propidium iodide (PI) positive cells. PI is excluded by the intact membrane of vital or apoptotic cells but penetrates the membranes of necrotic cells.[30]

Cell death from CPT involves caspase-3 activation and a slow transition (2 to 15 hours) from vital to apoptotic and then to necrotic cells; hence in our protocol anx-FH is present for the final 4 hours of the 16 hour incubation with CPT, or during the apoptotic to necrotic transition, see Figure 3a of [29] and [31]. Here we employed the HL-1, A549 and a CHO cell lines (versus the CHO, 786-0 and H9C2 used previously),[10] bringing to a total of 5 cell lines where annexin V nanoparticles (either anx-CLIO(Cy5.5) or anx-FH) are protective against CPT cell death.

Anx-FH also had protective effects when cells were exposed to the mechanically disruptive effects of a brief sonication (3 cycles, 5 minutes), with anx-FH then added for a two-hour incubation. Mechanical disruption produces cell damage that is so fast that it is independent of cell death pathways seen with agents like CPT that cause cell death by a slow caspase-3 activation, which leads to apoptosis followed by necrosis.[29] Anx forms a 2 dimensional array stabilizing the membrane against disruption and promotes membrane repair.[12] The high density of anx on FH further enhances the anx-FH cytoprotection that is not seen with either anx or FH alone.

In conclusion, we describe anx-FH, a nanoparticle of 28 nm with a surface consisting of 7.6 copies of a 36 kDa polyhis tagged annexin V's and which has a cytoprotective activity. The dimer of annexin, diannexin, is more active than annexin V monomer in part because of tighter binding to PS exposed on red blood cells.[13] The attachment of anx to FH yields anx-FH, a NP with far more anx's per mole than diannexin (7.6 versus 2). It will be of interest to compare anx monomers, anx dimers and anx-NP's in the various bioassays of cytoprotection, and to determine the protective effects of anx-FH in additional animal models of ischemia induced cell death.

Notes and references

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