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14. ABSTRACT Annexin V has been produced in good purity and high yield has been shown to bind strongly to plastic-immobilized phosphatidylserine (PS). Annexin V was conjugated with a suspension of single-walled carbon nanotubes (SWNTs) and carboxymethyl-cellulose (CMC) that had been dialyzed; this conjugation involves reaction of carboxyl groups of CMC with amino groups of the protein. A binding assay of this complex to human endothelial cells with PS exposed on the cell surface showed strong binding, indicating that the covalently bound annexin V is functional. Tests of laser treatment of endothelial cells on 24-well plates were carried out to determine the energy density range that would allow the cells to grow (≤ 360 J/cm ²). However, laser tests of endothelial cells on plates with the SWNT-CMC-annexin V complex present up to an energy density of about 200 J/cm ² did not show a conclusive cell-killing effect. It has subsequently been found that free CMC is not removed to a significant degree by dialysis, indicating that annexin V was probably conjugated mainly to free CMC. Therefore, two alternative methods to attach annexin V to a molecule adsorbed to SWNTs are currently being pursued.					
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INTRODUCTION

This project explores a novel treatment of breast cancer that uses single-walled carbon nanotubes (SWNTs) in photodynamic therapy (PDT). SWNTs are unique in that they strongly absorb near-infrared (NIR) light, while biological systems have relatively low levels of absorption of NIR light. In order to have a simple and fast means of delivery of the SWNTs to the tumor, it is proposed to target the SWNTs by conjugation with human annexin V, which has been shown to bind to phosphatidylserine (PS) exposed on the surface of endothelial cells in blood vessels in tumors; PS is not exposed on the surface of endothelial cells in blood vessels in normal organs. The purpose of this project is to produce a conjugate of SWNTs and annexin V, verify that this conjugate will bind specifically to surface-immobilized PS *in vitro* and to the surface of human endothelial cells *in vitro* in which PS has been induced to be on the cell surface, and demonstrate that endothelial cells with SWNT-annexin V bound can be killed by NIR light. The scope of the project is to provide a demonstration of this concept to cells grown *in vitro*.

BODY

The research accomplishments of this project to date are the following:

Task 1 - Production of the SWNT-annexin V complex

Recombinant annexin V has been expressed in *Escherichia coli* and then purified to homogeneity using a process that we have developed previously. The methods for cloning, expression, and purification of annexin V are the same as we have used for other recombinant proteins (1). The yield of purified annexin V was excellent: 145 mg/liter of the starting culture broth. An SDS-PAGE electrophoresis gel of the purified protein showed only one band after staining with Coomassie blue. The first six amino acids of the amino-terminus of the protein were sequenced and found to be correct.

For the attachment of annexin V to SWNTs, we initially used the sodium cholate suspension-dialysis method that we found to work well for the model protein horseradish peroxidase (2). However, after the dialysis of the sodium cholate away from the annexin V in the presence of SWNTs and then centrifugation, this method resulted in complete precipitation of the SWNTs, meaning that it would not yield individually suspended SWNTs with annexin V adsorbed.

For stable attachment of annexin V to SWNTs, we have focused on a method in which the protein is covalently attached to a molecule that is strongly adsorbed to the SWNTs. We have avoided conjugating the protein directly to the SWNTs, since this results in the SWNTs losing the desired strong absorbance in the NIR wavelength region (980 nm for the SWNTs we are using) (3). We have focused on using a method developed by Collaborating Investigator Daniel Resasco that has been successful in fully suspending SWNTs. In this method, the SWNTs are suspended using the polymer carboxymethylcellulose (CMC). We have been using CMC with a molecular weight of 50,000 Da and an average degree of substitution of 80% (i.e., 4 of 5 sugar groups have a carboxyl group). Then, we have reacted the carboxyl groups of the SWNT-CMC complex with primary amino groups of annexin V using EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride). The procedures that we have used for

preparing SWNT-CMC and for coupling annexin V to SWNT-CMC are given in the Appendix I. This procedure resulted in a stable suspension. The protein concentration was measured using the Bradford protein assay, and the SWNT concentration was measured from the absorbance at 800 nm. The results of this conjugation are given in Table 1.

Table 1. Results for the conjugation of annexin V to SWNT-CMC

Sample	Protein Concentration, mg/L	SWNT Concentration, mg/L	Protein Weight, mg/mg SWNT Weight
SWNT-CMC-annexin V suspension after centrifugation	74	14.6	5.1
Final dialysis solution (2 L) using 100 kDa membrane	0	—	—

After testing with this SWNT-CMC-annexin V complex showed that there was little effect of laser light at 980 nm on human endothelial cells *in vitro* when the complex was added to the cells, separate dialysis tests were performed with CMC at the same CMC concentration as in the protocol (Appendix I) but without SWNTs added. A 4 hour dialysis with a 100 kDa membrane showed that negligible CMC had crossed the dialysis membrane. This dialysis time should cause near equilibrium between the inside and outside of the dialysis bag. When a dialysis was done using a 300 kDa membrane with both CMC and SWNTs present at the same concentrations in the protocol (Appendix I), there was a significant loss of SWNTs through the dialysis membrane. Thus, it has been concluded that the use of CMC and SWNTs for attaching annexin V is problematic because of the difficulty in separating CMC and SWNTs.

Therefore, work has recently been initiated to investigate two alternative methods to attach annexin V to a molecule adsorbed to SWNTs:

1. Suspend the SWNTs by adsorbing 1-pyrenebutanoyl succinimide to the SWNTs and then react this complex with primary amino groups of annexin V to form a stable amide linkage. There have been reports from three research groups about the use of this method (4-6). Chen et al. (4) report that the use of this method leads to a complex that is highly stable against desorption in aqueous solutions. After the adsorption of 1-pyrenebutanoyl succinimide, Shao et al. (6) added polyethylene glycol (PEG, molecular weight 8000) to cover unoccupied sites on the SWNT so that undesirable binding with other biomolecules would be prevented.
2. Adsorb the bile salt deoxycholate to the SWNTs and then react the carboxyl groups of the SWNT-deoxycholate complex with primary amino groups of annexin V using EDC to form a stable amino linkage. Collaborating Investigator Daniel Resasco has successfully used deoxycholate to suspend SWNTs, and other investigators have found deoxycholate to be

effective in suspending SWNTs (7, 8). The fact that deoxycholate is a bile salt means that it will be biocompatible when used in the bloodstream. Prior to the conjugation of annexin V to the SWNT-deoxycholate complex, an experiment will be performed to determine if annexin V will displace the deoxycholate adsorbed to the SWNTs, which occurred with adsorbed sodium cholate. If this is the case, then this method would not be appropriate, since a SWNT-deoxycholate-annexin V complex would be designed to be injected into the bloodstream, where it would interact with proteins in the serum; these serum proteins would displace the deoxycholate-annexin V conjugate, which would mean that the SWNTs would not reach their desired target.

Task 2 – Test of the function of the SWNT-annexin V complex

For the binding studies, the annexin V was labeled with biotin. Ran et al. previously labeled annexin V for use in both *in vitro* and *in vivo* binding studies; the biotinylation procedure did not impair the ability of annexin V to bind to phosphatidylserine (9). For this labeling, a kit obtained from KPL was used; the procedure used is given in Appendix II.

The biotinylated annexin V was tested for its ability to bind to PS immobilized on plastic microtiter plates using the procedure given in Appendix III, which is similar to a procedure used by Ran et al. (9). The results of this binding assay are shown in Figure 1, which also indicates the value of the dissociation constant (K_d) and the goodness of fit of the data (R^2). The dissociation constant of 5.1 nM indicates strong binding of annexin V to PS, which shows that the annexin V is functional. Prism 5 software (GraphPad Software, San Diego, CA) was used in the calculation of K_d here and in later binding experiments. For this calculation, it was necessary to make a correction to the original concentration of annexin V for the amount that bound to the plate. The concentration on the x-axis in Figure 1 represents the concentration of annexin V in equilibrium with the annexin V bound to the plate.

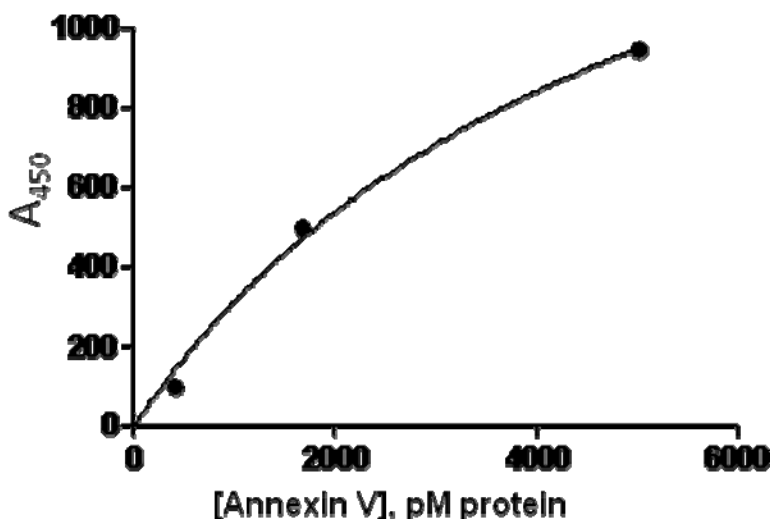


Figure 1. Data for annexin V binding to PS immobilized on plastic microtiter plates. $K_d = 5.1$ nM and $R^2 = 0.992$.

The biotinylated annexin V was then coupled to SWNT-CMC using the procedure given in Appendix I, and a binding assay of the SWNT-CMC-annexin V complex to human endothelial cells *in vitro* was performed in which PS is exposed on the surface of the cells by the addition of a low level of hydrogen peroxide (1 mM). The procedures for the culturing of the endothelial cells are given in Appendix IV, and the procedure for the assay of the binding of SWNT-CMC-annexin V to endothelial cells is in Appendix V. In the culturing of the endothelial cells, it was found to be important not to grow the cells to complete confluence, otherwise the cells would not come off the plate when treated with trypsin and could not be passaged further. The results of this binding assay are shown in Figure 2. The dissociation constant of 2.9 nM indicates strong binding of SWNT-CMC-annexin V to PS, which shows that the covalently bound annexin V is functional.

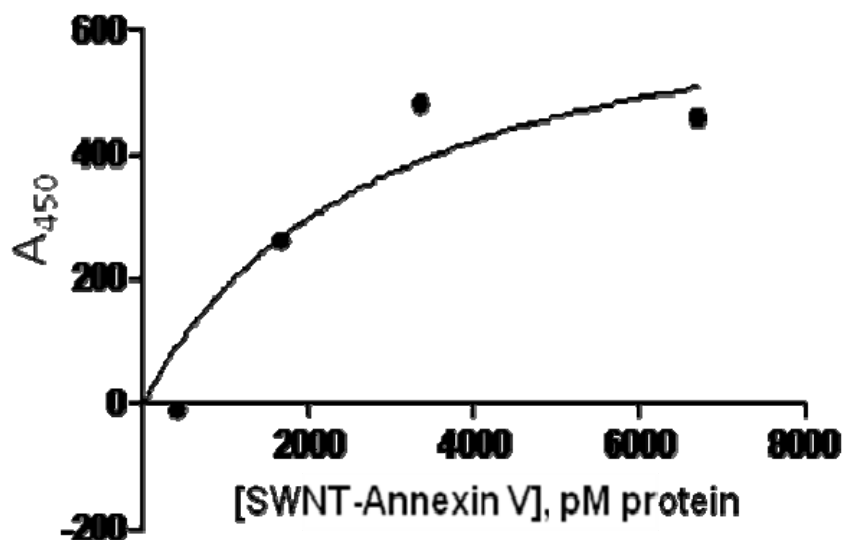


Figure 2. Data for SWNT-CMC-annexin V binding to PS immobilized on plastic microtiter plates. $K_d = 2.9$ nM and $R^2 = 0.864$.

Before the work on biotinylation of annexin V, we tried to measure the binding of annexin V using an antibody to annexin V for detection. The procedure was the same as in Appendix III, except for steps 7 and 8 a rabbit anti-annexin V antibody was added for 2 hours, the plate was washed, a goat antirabbit IgG antibody conjugated to horseradish peroxidase (HRP) was added for 2 hours, and the plate was washed again. This assay gave inconsistent results and only small changes in A_{450} as the concentration of annexin V increased. We have concluded that the antibody binding site on annexin V may have been blocked once annexin V was bound to the plate.

Task 3 – Test of the effect of NIR light on endothelial cells with SWNT-annexin V bound

These tests were performed with a Sharplan laser set at a wavelength of 980 nm (LaserCare 50, Sharplan Medical Systems). This laser has an effective maximum power of

approximately 35 watts. The size of the beam can be changed by adjusting the distance from the end of the laser fiber to the bottom of the plate. The tests were done on cells grown in 24-well plates (see Appendix IV for the procedure for cell culturing). Depending on the power level, the laser beam had a diameter of 2.2 cm for a single well or 5.0 cm for four adjacent wells. For each laser condition (power and time), three wells were treated.

The metabolic activity of the cells before and after laser treatment was evaluated by the Alamar Blue assay. Alamar Blue (or resazurin) is oxidized, blue, and nonfluorescent and is reduced to a pink fluorescent dye in the medium by cell activity (10). There is a direct correlation between the reduction of Alamar Blue in the growth media and the quantity/proliferation of living organisms, ranging from bacteria to mammalian cells. The Alamar Blue assay is advantageous since Alamar Blue is nontoxic to cells and does not kill the cells to obtain measurements, as does the MTT assay. The procedure for using this assay is given in Appendix VI (steps 16-19).

Tests were performed to determine the laser energy density (J/cm^2) that could be used without harming normal cells. The procedure used is given in Appendix VI, and the results are shown in Figure 3. At the highest energy level, the power density was at $9.4 \text{ W}/\text{cm}^2$ for 60 sec. There was a significant drop in the relative fluorescence units (RFU) at the highest energy level ($p < 0.05$ by the two-tailed t-test). These results indicate that the laser light does not significantly affect the ability of cells to grow at or below $360 \text{ J}/\text{cm}^2$.

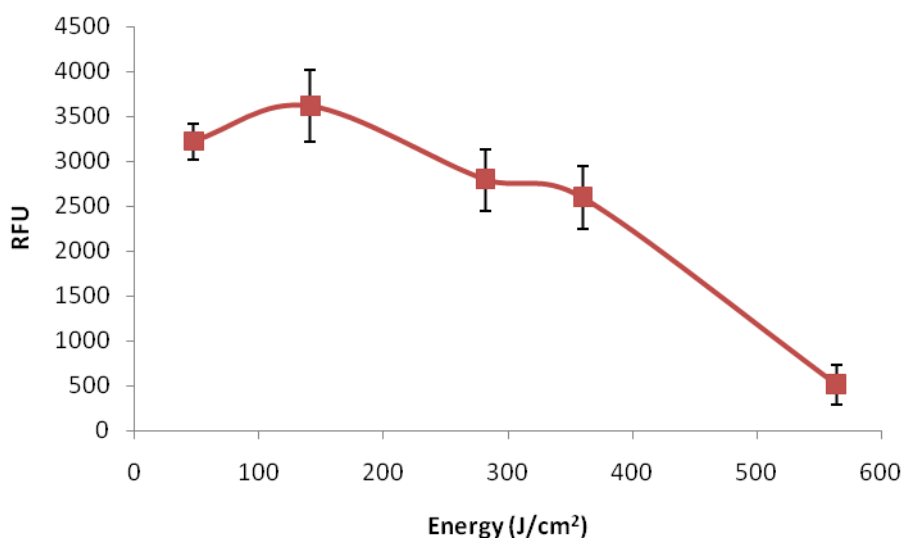


Figure 3. Effect of laser light at 980 nm on human endothelial cells grown in 24-well plates. RFU is relative fluorescence units (mean $\pm$ SD).

For the test of the effect of the addition of the SWNT-CMC-annexin V complex to the endothelial cells and subsequent treatment with laser light at 980 nm, the procedure in Appendix VI was used, except the SWNT-CMC-annexin V complex was added in step 9 at a concentration of 20 nM. The energy density was limited to about $200 \text{ J}/\text{cm}^2$ in order not to harm cells not treated with SWNT-CMC-annexin V (control). The results are shown in Figure 4. At the

highest energy level, the power density was 1.5 W/cm^2 for 135 sec. The differences between cells with and without SWNT-CMC-annexin V at the two highest energy density levels were confirmed by cell counting. Only at an energy level of 136 J/cm^2 was there a significant difference in RFU between cells with and without SWNT-CMC-annexin V ($p < 0.05$ by the two-tailed t-test). Therefore, the Alamar Blue assay results do not indicate a conclusive effect of the combination of laser light and the SWNT-CMC-annexin V complex. Cells with and without the presence of the SWNT-CMC-annexin V complex were observed by a microscope, and no differences were observed (i.e. the cells in the presence of the SWNT-CMC-annexin V complex did not appear darker because of the black SWNTs bound). These findings indicate that annexin V was probably conjugated mainly to CMC. See Task 1 for a discussion of current work being initiated to attach annexin V to a molecule adsorbed to SWNTs.

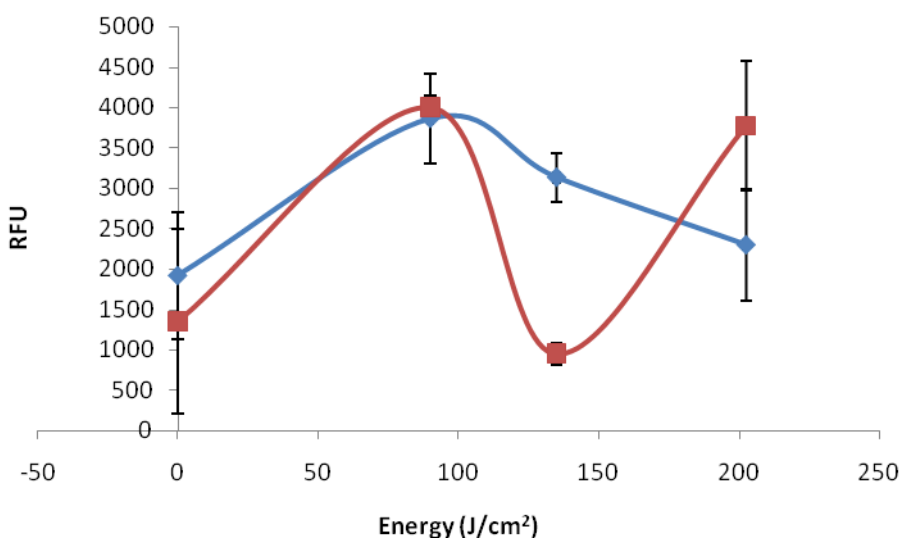


Figure 4. Effect of laser light at 980 nm on human endothelial cells grown in 24-well plates with (squares) and without (diamonds) the addition of SWNT-CMC-annexin V. RFU is relative fluorescence units (mean $\pm$ SD).

KEY RESEARCH ACCOMPLISHMENTS

1. Purified recombinant annexin V was produced in good purity and high yield.
2. The purified annexin V strongly binds to plastic-immobilized PS as indicated by its dissociation constant (5.1 nM).
3. The purified annexin V retains its strong binding to PS on human endothelial cells after it is conjugated to SWNT-CMC, as indicated by its dissociation constant (2.9 nM).
4. The energy density range that can be used for treatment of human endothelial cells *in vitro* without significantly affecting the ability of the cells to grow was determined.

REPORTABLE OUTCOMES

Harrison, R.G., and Resasco, D.E., “Composition and Method for Cancer Treatment Using Targeted Single-Walled Carbon Nanotubes,” U.S. Patent Application, February 19, 2008.

Palwai, N.R., Martyn, D.E., Resasco, D.E., Neves, L.F.F., Tan, Y., and Harrison, R.G. (speaker), “Attachment of Proteins to Single-Walled Carbon Nanotubes for Targeting and Treatment of Tumors,” AIChE Annual Meeting, Salt Lake City, November, 2007.

Harrison, R.G. (invited speaker), “Novel Fusion Proteins Designed for Targeted Therapies,” Cambridge Healthtech Institute’s Fourth Annual Conference on Peptide and Protein-Based Therapeutics, San Diego, CA, January 7, 2008.

Neves, L.F.F., Lazrak, Y., Tran, K., Martyn, D.E., Resasco, D.E., and Harrison, R.G., “Endothelial Cell Binding Properties and Absorption Spectra of Single-Walled Carbon Nanotubes with Human Protein Annexin V Attached,” poster presentation at the 37th Annual Biochemical Engineering Symposium, Iowa State University, Ames, Iowa, April 26, 2008.

Neves, L.F.F., Lazrak, Y., Martyn, D.E., Resasco, D.E., and Harrison, R.G. (presenter), “Single-Walled Carbon Nanotubes Targeted to the Tumor Vasculature for Breast Cancer Treatment,” poster presentation, Department of Defense Era of Hope Breast Cancer Research Meeting, Baltimore, June 26, 2008.

“Carbon Nanotube Technology Center (CANTEC),” DOE, D.E. Resasco, PI, R.G. Harrison, Co-PI, and five additional Co-PI’s, 8-1-08 to 7-31-10, \$960,000 (Title of project directed by R.G. Harrison: “Targeted Single-Walled Carbon Nanotubes in Photodynamic Therapy for Cancer.” The objective of this project is to study the effect of the SWNT-annexin V complex on an orthotopic human breast tumor xenograft in mice using i.v. injection followed by exposure of the xenograft to NIR light. There is no overlap of this project with the DOD Breast Cancer Research Concept Award, since the DOD award is for work only with cells *in vitro*.).

“Single-Walled Carbon Nanotubes Targeted for the Treatment of Pancreatic Cancer,” NIH Pilot Studies in Pancreatic Cancer (R21), R.G. Harrison, PI, 4-1-09 to 3-31-11, \$275,000 direct costs, pending.

CONCLUSION

Recombinant annexin V has been produced in good purity and high yield, and it has been shown to bind strongly to plastic-immobilized PS (dissociation constant of 5.1 nM). Annexin V was conjugated with a suspension of SWNTs and CMC that had been dialyzed; this conjugation involves the reaction of carboxyl groups of CMC with amino groups of the protein. A binding assay of this complex to human endothelial cells with PS exposed on the cell surface showed a strong binding (dissociation constant of 2.9 nM). Tests of laser treatment of endothelial cells on 24-well plates were carried out to determine the energy density range that would allow the cells to grow ($\leq 360 \text{ J/cm}^2$). However, laser tests of endothelial cells on 24-well plates with the SWNT-CMC-annexin V complex present up to an energy density of about 200 J/cm^2 did not

show a conclusive cell-killing effect. It has subsequently been found that CMC is not removed to a significant degree by dialysis, indicating that annexin V was probably conjugated mainly to free CMC. Therefore, two alternative methods to attach annexin V to a molecule adsorbed to SWNTs are currently being pursued. In one method, 1-pyrenebutanoyl succinimide is adsorbed, and in the other one deoxycholate is adsorbed.

The proposed project has the potential for a revolutionary impact on the treatment of breast cancer. There would be significantly fewer side effects than for conventional PDT, because the light-sensitive agent (SWNTs) is targeted specifically to the tumor. This therapy would be much less invasive than surgery and could replace surgery in some cases, which would result in much less pain and disfigurement to breast cancer patients and much shorter stays in the hospital.

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APPENDIX I

Procedure for SWNT-CMC Preparation and for Two-Step Coupling of Proteins using EDC and NHS

A) SWNT-CMC preparation

1. Weigh out 3 mg of single walled carbon nanotubes;
2. Weigh out 140 mg of CMC (Sigma);
3. Weigh 7 mg of dH₂O and mix the solution;
4. Sonicate the suspension for 30 minutes at 22% of the total activity of the sonicator;
5. Centrifuge the suspension at 30,000 x g for 30 minutes;
6. Save 1 ml of the suspension for further NIR analysis.

B) Procedure for two-step coupling of proteins using EDC and NHS or Sulfo-NHS

1. Prepare a 2 L solution of 0.5 M NaCl;
2. Put the SWNT-CMC suspension into a 100 kDa membrane in order to remove the unbound CMC (CMC has an approximate M.W. of 50 kDa);
3. Perform the dialysis at 4 °C for 8 h with constant stirrer agitation (Change the buffer after 4 h.);
4. Allow the EDC and NHS bottles to equilibrate at room temperature;
5. Add 4.9 mg of EDC (~ 2 mM) and 7.35 mg of NHS of 12.25 ml (final volume of dialysis of 12.25 ml) of SWNT-CMC solution and react for 15 minutes at room temperature;
6. Add 17.15 µl of 2-mercaptoethanol (final concentration of 20 mM) to quench the EDC;
7. Add protein annexin V (0.345 mg/ml) with SWNT-CMC. Allow the solution to react for 2 h at room temperature;
8. To quench the reaction, add the hydroxylamine to a final concentration of 10 mM. This method hydrolyzes nonreacted NHS present on SWNT-CMC and results in regeneration of the original carboxyls. Other quenching methods involve adding 20-50 mM Tris, lysine, glycine or ethanolamine; however, these primary amine containing compounds modify carboxyls on CMC.
9. Remove excess reagent using a dialysis membrane (100 kDa) immersed in a 2 L sodium phosphate buffer (20 mM pH 7.4).
10. Replace the buffer after 4 h from the beginning of the dialysis, which has a total duration of 8 h.
11. Measure the final volume of the solution.
12. Centrifuge the solution at 29,600 x g for 1 h, in order to isolate the SWNT-CMC fraction bound to annexin V.
13. Measure the protein concentration using the Bradford protein assay.

APPENDIX II

Procedure for the Labeling of Annexin V with Biotin (SureLINK Biotin Kit from KPL)

This protocol describes the conjugation reaction for 1 ml 1X Modification Buffer (100 mM phosphate, 150 mM NaCl, pH 7.2-7.4) at 0.2-5.0 mg/ml.

1. Prepare the modification buffer, diluting the 10X Modification Buffer in molecular biology grade water to 1X.
2. Dissolve SureLINK Chromophoric Biotin in DMF immediately prior to use. Tap down and equilibrate the biotin vial to room temperature prior to opening to avoid moisture condensation. The NHS-ester moiety of SURELINK chromophoric biotin is hydrolyzed when exposed to water. Note that extended storage of the biotin/DMF solution is not recommended. Aliquots may be stored at -20 °C under desiccation for short periods of time.
3. Rehydrate or transfer the protein annexin V into 1 ml 1X Modification Buffer (100 mM phosphate, 150 mM NaCl, pH 7.2-7.4) at 0.2-5.0 mg/ml. We will **use 1 mg of protein** (since that is the lowest amount that is possible to measure accurately). For the labeling of proteins at a concentration of **1 mg/ml**, KPL recommends a **40-fold molar excess** of biotin for conjugates that will be used in ELISA.

Buffers containing Tris, imidazole, glycine or primary amines should not be used due to competition with the conjugation reaction. If the sample is stored in one of these buffers, remove by buffer exchange against 1X Modification Buffer. PBS (10 mM phosphate, 150 mM NaCl, pH 7.2-7.4) is not recommended due to poor buffering capacity.

4. Immediately prior to use, prepare a 20 mg/mL (25 nmole/ μ L) stock solution of SureLINKTM Chromophoric Biotin in anhydrous DMF.

SureLINK Chromophoric Biotin	DMF to prepare a 20 mg/ml of chromophoric biotin
0.5 mg	25 μ l
1.0 mg	50 μ l
10.0 mg	Variable, depending on the weighed amount

(Since we ordered the kit that provide us 1 mg aliquots, the required volume of DMF will be equal to 50 μ l).

Tap down and equilibrate biotin vial to room temperature prior to opening to avoid the condensation of moisture. The NHS-ester moiety of chromophoric biotin is hydrolyzed when exposed to water.

If high biotin molar excess is chosen for a highly concentrated protein solution, the concentration of biotin stock solution can be increased to 40 mg/mL (see Calculation section for more details).

5. Add an appropriate volume of 20 mg/mL SureLINK™ Chromophoric Biotin to the antibody or protein solution. **The volume of chromophoric biotin to use is equal to 4.44 µl (if we use a molar excess ratio of 40) or 2.22 µl (using a molar excess ratio of 20).**

See the Calculation section Part 2 to determine the appropriate volume of SureLINK™ Biotin. The solution may become cloudy, which does not affect the labeling reaction. The percentage of biotin solution added should be less than 5% of the total reaction volume to minimize protein precipitation by DMF.

Examples for Conjugation Reactions: When antibodies at 1 mg/mL are used for labeling, a 40-fold molar excess of biotin is recommended for ELISA and a 10-fold molar excess of biotin is recommended for Western blotting.

Volume of IgG solution (µL)	Protein conc. (mg/mL)	Total IgG amount (mg)	Biotin molar excess (fold)	Volume of 20 mg/mL biotin stock (µL)	DMF % in reaction mixture
1000	0.2	0.2	60X	3.2	0.3
1000	0.5	0.5	60X	8.0	0.8
1000	0.5	0.5	40X	5.3	0.5
1000	1	1.0	40X	10.7	1.1
1000	1	1.0	10X	2.7	0.3
1000	4	4.0	40X	42.7	4.3
1000	4	4.0	10X	10.7	1.1

When we use a 40-molar ratio and a 20-molar ratio, the % DMF is equal to 0.442 % and 0.220 % respectively.

6. Incubate at room temperature for two hours with gentle agitation. Longer incubation times, up to overnight, may be used. However, microbial growth and protein degradation may be a concern.
7. Remove the unconjugated chromophoric biotin by spin filter.

KPL recommends the following desalting procedure using the spin filters included in the kits:

- a. Add the biotinylated protein solution to the top section of the spin filter. Each spin filter can hold up to 500 μ L. Concentrate the sample by **centrifugation at 12,000 x g for 30 minutes**. The concentrated solution will remain in the top section of the spin filter. Dispose of the filtrate (bottom section) of the spin filter.
- b. Repeat as necessary until a buffer exchange of at least 200-fold is achieved.

Desalting columns or dialysis cassettes can also be used to remove unconjugated biotin. Choose an appropriate molecular weight cutoff (MWCO) for your protein.

8. The biotinylated protein is now ready to use.

APPENDIX III

Procedure the for the Binding of Annexin V to Plastic-immobilized Phosphatidylserine

1. After labeling the 1 mg of annexin V using the SureLINK Biotin Kit, the phospholipids that were received in chloroform are diluted further in chloroform to a concentration of 50 µg/ml.
2. One-hundred µl of above solution was added to wells of 96-well plates (Corning propylene plates, product number 3359).
3. After evaporation of solvent in air (used laminar flow hood), plates were blocked at room temperature for 2 hours with 0.5 % BSA diluted in PBS (phosphate buffered saline) containing 2 mM Ca^{2+} (binding buffer). Remove the binding buffer after the blocking, without washing the plate.
4. Biotinylated annexin V conjugate was diluted in binding buffer at an initial concentration of 6.7 nM. Serial dilutions of this solution were prepared and 100 µl was added to plates.
5. The plates were incubated at room temperature for 2 h.
6. The plate was washed (4x) with 200 µl of PBS containing 2 mM Ca^{2+} (washing buffer) per well at room temperature.
7. After washing, incubate with HRP-Streptavidin for 1 hour at room temperature. According to the datasheet from KPL the suggested concentration for ELISA is 2 µg/ml.
8. The plate was washed (4x) with 200 µl of PBS containing 2 mM Ca^{2+} (washing buffer) per well at room temperature.
9. Prepare the OPD solution.
10. 200 µl of O-phenylenediamine (OPD) solution (chromogenic substrate) was used to detect the Annexin V.
11. Wait 30 minutes at room temperature in the dark, in order to minimize the color change of the OPD.
12. Read the plates at 450 nm with the Biotek KC4 microtiter plate reader. For the blank, use the same procedure with annexin V not added.

APPENDIX IV

Procedures for Culturing Endothelial Cells

Cell type: Human endothelial cells, ATCC catalog number CRL-2472 (Human Aorta Endothelium)

Media: F12K with 10% BFS, 5 mL of antibiotic (penicillin and streptomycin), 3 mL of heparin (0.1 mg/ml) and 1.5 mL of ECGS (endothelial cell growth supplement (0.03 mg/ml)

Culture of endothelial cells in T-75 flasks (from frozen vial)

- 1) The media was warmed at 37 °C (at least 30 min earlier).
- 2) The new T-75 flask was incubated with 1-2 mL of gelatin for at least 10 min.
- 3) The frozen vial was thawed in a water bath (37 °C) carefully so that the water did not touch the lip of the vial.
- 4) The gelatin solution was taken off from the flask, and 10 mL of media was added to the flask.
- 5) The cells from the vial were inoculated in the media. Media from the flask was added into the vial to recover all the cells and, then, this media returned to the flask.
- 6) The flask was incubated at 37 °C (0.5% of CO₂) until ready for use (approximately 2 weeks).

Culture of endothelial cells in 24-well plates

- 1) Cells were grown in T-75 flasks until 70% of confluence was reached.
- 2) The media and the trypsin were warmed at 37 °C (at least 30 min before use).
- 3) The new 24-well plate was incubated with 0.1-1 mL of gelatin per well, for at least 10 min.
- 4) The media from the T-75 flask was removed and 2-3 mL of trypsin was added.
- 5) The flask was incubated for 5-15 min at 37 °C (0.5% CO₂), until all the cells detached from the surface of the flask.
- 6) To quench the reaction, 6-8 mL of media was added to the flask.
- 7) The cell concentration was determined with a hemocytometer.
- 8) 5×10^4 cells were added to each well.
- 9) Media was also added to each well so that the final volume was 1 mL.
- 10) The plate was incubated at 37 °C (0.5% CO₂) until use (70% confluence).

Media for endothelial cells

F12K media (1 flask, 500 mL)

FBS (fetal bovine serum) 50 mL

Antibiotic (penicillin and streptomycin) 5 mL

Heparin 3 mL

ECGS 1.5 mL

Keep at 4 °C. Warm for 30 min at 37 °C before use.

APPENDIX V

Assay for the Binding of SWNT-CMC-annexin V on Externally Positioned PS on the Surface of Endothelial Cells

1. Grow cells using F12K media containing 10% FBS until they reach 85 % confluence in T-75 flasks.
2. Count the cells using hemacytometer.
3. Transfer cancer cells (5×10^4) to 24 well plates and grow until 85 % confluence is reached.
4. PS was exposed on the surface of cells by the addition of hydrogen peroxide (1 mM). Treat cells with 100 μ l wash/binding buffer containing the F12K media containing 1 mM of H_2O_2 for 1 h at 37 °C and 5 % CO_2 .
5. Fix the cells by adding 100 μ l PBS buffer containing 0.25 % glutaraldehyde and Ca^{2+} (2 mM).
6. Quench excess aldehyde groups incubating with 50 mM of NH_4Cl (100 μ l) diluted in PBS buffer containing Ca^{2+} (2 mM) for 5 min.
7. Dilute the SWNT-annexin V conjugate in 0.5 % BSA diluted in PBS buffer and Ca^{2+} (2 mM) with an initial concentration of 6.7 nM.
8. Do serial 2-fold dilutions of this concentrated fusion protein solution until final concentration of 6.7 pM.
9. Add SWNT-CMC-annexin V (300 μ l) to wells in the increasing concentration of SWNT-CMC-annexin V.
10. For each concentration of SWNT-CMC-annexin V, the experiment is done in triplets.
11. Incubate for 2 h.
12. Wash plates with 0.5 % BSA diluted in PBS buffer and Ca^{2+} (2 mM) (300 μ l).
13. Add 300 μ l of streptavidin-HRP (2 μ g/ml) and incubate for 1 h at room temperature.
14. Wash the plate with PBS (300 μ l).
15. Add the chromogenic substrate O-phenylenediamine (OPD, 300 μ l).
16. Wait for 30 min and transfer 100 μ l of the supernatant to 96-well plates.
17. Measure absorbance at 450 nm.

APPENDIX VI

Test of the Effect of Laser Light at 980 nm on Human Endothelial Cells *In Vitro*

1. Grow cells using F12K media containing 10% FBS until they reach 85 % confluence in T-75 flasks.
2. Count the cells using hemocytometer.
3. Transfer cancer cells (5×10^4) to 24 well plates and grow until 85 % confluence is reached.
4. Perform the Alamar Blue assay (see steps 16-20) the day before the laser tests. This assay will be used as a measure of the number of cells in each well, and the results will be used to standardize subsequent Alamar Blue results based on the same number of cells in each well.
5. Warm up the media in the incubator at 37 °C.
6. Remove the media from the wells.
7. PS was exposed on the surface of cells by the addition of hydrogen peroxide (1000 μ M). Treat cells with 300 μ l binding buffer containing the F12K media and containing 1 mM of H_2O_2 for 1 h at 37 °C.
8. Wash plates 1x with F12K media (containing 10% FBS) (300 μ l).
9. Add F12K media plus 2 mM Ca^{2+} (300 μ l) to the wells.
10. For each plate, the experiment is done in triplets.
11. Incubate for 2 h in the incubator.
12. Wash plates 4x with F12K media (containing 10% FBS plus 2 mM Ca^{2+}) (300 μ l).
13. Add 300 μ l of F12K media with 2 mM Ca^{2+} to the wells.
14. Perform power measurements using a beam with a diameter of 5 cm for four adjacent wells in square pattern or 2.2 cm for single wells.
15. Perform the laser test.
16. Evaluate the cell viability 1 hour later by adding Alamar Blue in an amount equal to 10% of culture media (30 μ l of Alamar Blue + 300 μ l of media) volume to the wells. The Alamar Blue will be added to the plates at once.
17. Incubate for 4 hours.
18. Transfer the samples (300 μ l/well) to a 96-microtiter plate.
19. Measure fluorescence at 590 nm (using excitation at 530 nm). Use a well containing only media with Ca^{2+} as a blank.
20. Add 300 μ l of fresh media to each well on the 24-well plate. Treat the cells with trypsin that are attached and combine with the cells in the 96-well plates and count the cells in each well.