Focused Ultrasound and Poly(2-ethylacrylic acid) Act Synergistically To Disrupt Lipid Bilayers in Vitro

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The delivery of macromolecules such as proteins and DNA to the cytosol of targeted cells remains a significant problem in gene therapy and chemotherapy. Such macromolecules can be efficiently introduced into targeted cells through the process of receptor-mediated endocytosis.[1] Unfortunately, these biomolecules are then usually trafficked via endosomes to lysosomes, where they are degraded by enzymes.[2] A method is needed to efficiently release endocytosed macromolecules into the cytosol before they merge with lysosomes and lose their bioactivity.

Endosomes have proton pumps that reduce their pHs to 5.0−6.5. It is possible to selectively disrupt endosomes by using agents that permeabilize their lipid bilayers only at such acidic pHs.[2] We have previously demonstrated that certain pH-sensitive synthetic polymers such as poly(2-ethylacrylic acid) (PEAA) cause efficient, pH-sensitive hemolysis[3] and also that they retain this action when they are chemically conjugated to streptavidin, a model for protein-based therapeutic agents.[4] This occurs because under mildly acidic conditions, where protonated, PEAA becomes relatively hydrophobic. PEAA has the ability to permeabilize lipid bilayer membranes through the creation of pores and channels.[5,6] This effect is directly proportional to the number of polymer molecules that interact with the cell membrane and therefore depends on the concentration of the PEAA[3] as well as pH.

Focused ultrasound has been shown to transiently permeabilize skin,[7,8] the blood−brain barrier,[9,10] and cell membranes.[11,12] This attribute has led to recent work using focused ultrasound for targeted drug delivery,[13,14] for transfection,[15−18] and for a number of other applications.[19] This previous work motivated us to investigate the ability of focused ultrasound and PEAA to synergistically permeabilize lipid bilayers. Other motivators include the fact that ultrasound and PEAA do so separately and that ultrasound has been observed to work synergistically with a variety of chemicals, as reviewed elsewhere.[19]

In the present study, human red blood cells (RBCs) were used as the model membrane system and hemolysis as the measure of membrane permeabilization. Briefly, isolated RBCs were suspended in a phosphate-buffered saline (PBS) solution with a pH of either 6.1 or 7.4 at a concentration of 2×10^8 cells/mL and a temperature of 37 °C. Ten micrograms of PEAA (synthesized as described elsewhere[3]) with a molecular weight of 25 kDa representing 2.38×10^{14} molecules of PEAA was placed in a 10 μL PBS solution, which was then added to the RBC suspension. Within minutes, focused ultrasound was applied as described elsewhere[20] to the resulting PEAA/RBC mixture. The acoustic protocol consisted of 150 pulses of 1.1 MHz ultrasound, each with a pulse length of 10 ms, applied once per second. The pulses had a spatial average, temporal average peak intensity of 2200 W/cm^2. This acoustic protocol applied in the absence of PEAA did not produce statistically significant hemolysis, nor did it warm the blood, as measured with a thermocouple (type K, Omega Engineering, Inc., Stamford, CT). After the ultrasound was applied to the PEAA/RBC solution, the solution was left at 37 °C to complete the hour that started with the introduction of PEAA. After that hour, the percent hemolysis was measured by spinning the RBC and PEAA suspension in a microcentrifuge (Eppendorf, 5410, Westbury, NY) at 14 000 rpm for 2 min and then measuring the hemoglobin content of the supernatant with a spectrophotometer at 541 nm. The results were then normalized against complete hemolysis achieved by mixing the RBC solution with distilled and deionized water. All of these results were compared with the hemolysis induced (a) by ultrasound alone, (b) by 10 μg of PEAA alone in RBC suspensions with a pH of 6.1, and (c) in control samples that were exposed to neither ultrasound nor PEAA. All experiments were performed in triplicate.

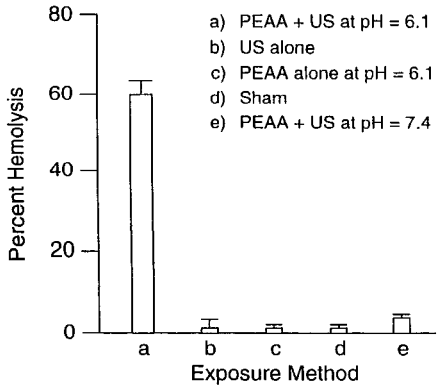

Figure 1. Percentage of red blood cell (RBC) disruption created by (a) poly(2-ethylacrylic acid) (PEAA) and ultrasound (US), (b) US alone, (c) PEAA alone, (d) by simply handling the cells; for all of these cases, the host fluid had a pH of 6.1. (e) As above, but for PEAA and ultrasound applied to a dilute suspension of RBC at a pH of 7.4. All experiments were performed in triplicate. The graphs show the mean and standard error. Note that the result in (a) differs from the other results in a statistically significant manner ($p < 0.05$).

The results, shown in Figure 1, demonstrate that PEAA and ultrasound act synergistically at reduced pH in vitro. Focused ultrasound applied to RBCs in the presence of PEAA at a pH of 6.1 produced 60% hemolysis of 10^8 cells (Figure 1a). This result is significantly greater ($p < 0.05$) via a two-tailed Student's *t*-test than the hemolysis achieved by ultrasound alone (2%, Figure

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1b), by this concentration of PEAA alone at a pH of 6.1 (2%, Figure 1c), or by simply handling the RBC (1%, Figure 1d).

Most studies[19] of ultrasound−drug synergy in biological systems use an ultrasound protocol that induces cavitation (the production and/or stimulation of dynamically active microbubbles[21]). Here, our ultrasound protocol alone does not induce significant cavitation[22] when applied to RBCs at a pH of 6.1. However, when both ultrasound and PEAA were applied to RBCs at a pH of 6.1, cavitation was observed.[22] It therefore appears that the synergy between ultrasound and PEAA at pH = 6.1 occurs at least because PEAA molecules at this pH can act as cavitation nucleation sites[23] or modify existing nucleation sites,[24,25] i.e., by creating gas pockets or enhancing existing gas pockets on impurities present in the fluid[26] that can be activated by ultrasound. This is consistent with published observations that demonstrate that PEAA reduces interfacial tension at low pH.[27] To test this hypothesis, consider Figure 1e, which shows that ultrasound applied to the PEAA/RBC solution at a pH of 7.4 produced negligible hemolysis—4%—as well as negligible cavitation.[22] Since at this pH PEAA is hydrophilic, this result supports the hypothesis that PEAA at a pH of 6.1 acts as a cavitation−initiation site, or modifies existing nucleation sites, in a manner consistent with what is known of natural cavitation nuclei. Also, the contrast in levels of hemolysis created by ultrasound and PEAA at a pH of 6.1 vs at a pH of 7.4 shows that the pH-dependent ability of PEAA at high concentrations alone to disrupt lipid bilayers[3] carries over to its application at low concentrations with ultrasound.

How may pH-dependent cavitation induction by PEAA generate the observed synergy between PEAA and ultrasound? One hypothesis is that polymer enhancement of cavitation induces direct membrane lysis by localized mechanical stresses associated that cavitation. Along the same lines, one may hypothesize that ultrasound, via cavitation, may weaken the cell membranes without significant lysis so that those membranes are more amenable to attack by the low concentration of PEAA used in the present study. In particular, ultrasound may generate transient pores or defects in the lipid bilayer, through which PEAA molecules could then more readily partition into the lipid bilayer. Additionally, one may hypothesize sonochemical enhancement[19] of the action of PEAA by the cavitation, as has been observed by others for other chemicals.[13,14] Further work is necessary to ascertain the veracity of these various hypotheses. Indeed, it is quite possible that many if not all of the proposed mechanisms play a role in the observed synergy between ultrasound and PEAA.

The present results, the ability of PEAA to be chemically conjugated to model therapeutic macromolecules,[4] and recent in vitro[28] and in vivo[29] observations of ultrasound's ability to acoustically activate bubbles that are within cells suggest that application of focused ultrasound and PEAA has the potential to enhance the delivery of endocytosed therapeutic macromolecules.

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