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Nano Engineering of Artificial Granulocytes for Cancer Diagnosis and Therapeutics

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The study of nanomedicine research, to date, has been concentrating on developing nanovectors for medical imaging, drug/gene delivery, and cell targeting, particularly in cancer diagnosis and therapeutics. Although quite successful in some areas, critical challenges remain, especially in the clinical settings. Some of the critical issues include delivery inefficiency, targeting non-specificity, and low uptake of theranostic-payloads by the cancerous lesions upon intravenous injection. Therefore, an alternative approach may be possible, via nanotechnology, by simulating some of the immune cells. In this *Technical Note*, we propose using specifically designed nanoparticles to simulate neutrophils that are capable of effective cancer cells targeting and killing without the complications in drug loading, biomarker conjugation, and assembly of chemical and physical therapeutic means. The simulated artificial cells mimic neutrophils, namely, granulocytes, according to their biological characteristics, for instance, positively-charged cell surfaces and the ability to release perferin upon binding onto the cancer cells leading to cytolysis. This *Technical Note* is intended to deliver a new concept in artificially-engineered granulocytes for cancer diagnosis and therapeutics.

Keywords: Artificial cells; neutrophils; engineered granulocytes; cancer therapeutics; nanomedicine.

1. Introduction

Simulation of biological cells has been a major undertaken in nanotechnologies. Based on general functions and behaviors of biological cells, various nanoparticle systems have been designed, synthesized, and developed for medical diagnosis and therapeutics. Studies have shown artificial cells can be engineered that mimic a biological entity. This concept has been extended to simulation of viruses for gene delivery in order to avoid their aggressive and invasive behaviors.[1,2] The most popular nano systems designed for medical theranostics are nano-scale polymer particles such as liposomes, polyethylene glycol (PEG), and poly(lactic-co-glycolic acid) (PLGA).[3,4] However, these types of nano systems are often referred to "nanocarriers" as their specific functions are mainly intended for encapsulating/releasing of drugs/genes for cancer therapeutics, decorating of fluorescent agents for medical imaging, and conjugating with the tumor-specific ligands for cell targeting.[5–7]

In cell-mimicking of an artificial entity, nanotechnologies can be utilized to serve the similar purposes, for instance, cancer cell targeting and

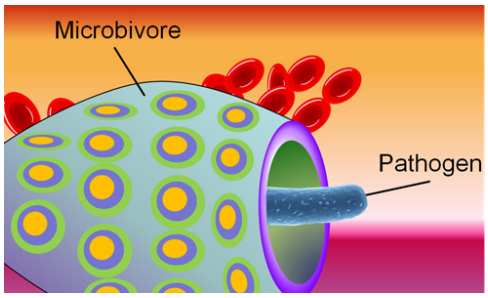

Fig. 1. Schematic diagram of microbivore.[8]

killing, but with strategically different approaches. One example is the engineered nanorobots that have attracted significant attention recently in the life science and engineering communities. A nanorobot can mimic certain white cells and deliver the designated functionalities, but with certain controlled risks. Amongst them, *Microbivore* is one such artificially designed mechanical phagocyte, proposed by Robert A. Freitas. Jr.[8] One may think of it as a micron-sized submarine with a self-propelling engine capable of operating independently in fluid (as shown in Fig. 1). For its submarine-like maneuverability, a special spheroidal shape is acquired so that it is able to pass through human capillaries of certain dimensions. However, as its name suggests, a *microbivore* is designed mainly to initiate phagocytosis that eliminates pathogens. In operation, it is powered by engulfing trapped microbes as fuels to support the cellular processes. As a concept, microbivore has inspired the design and development of artificial cells for medical diagnosis and therapeutics.

As a conceptual design, this *Technical Note* presents a model of an artificial cell, based on the characteristics of a type of white blood cells, namely granulocytes[9] by simulating its operational functionalities with the nano-scale particles. The simulated granulocytes may function not only to target tumor cells, but also effectively kill them off by various physical, chemical, or biological means. A new interpretation of immunological reaction is proposed for granulocytes to specifically bind onto the cancer cells in the vicinity via sheer electrostatic interactions. In this manner, the granulocytes and cancer cells will be moving towards each other, driven largely by Coulomb force attracted by opposite charges between granulocytes (positive) and cancer cells (negative). If such a hypothesis holds true, we will be able to understand the origin of "immune-response" from the viewpoint of cell electric charge, based on which new classes of artificial cells can be engineered.

2. Natural Cancer Cell Killers: Granulocytes

The immune system is essential for human survival, and therefore established with complicated biological structures that fence off foreign invaders such as bacteria, viruses, and parasites. The interaction is very much like fighting a war, with different levels of defenses; each layer is entrenched with different degree of immune specificity (enemy recognition).[10] The first layer often attacks bacteria and viruses in a "shotgun" fashion for its non-specific, but fast responses. However, a second layer is needed once the invaders penetrate the first one, which is defined as the adaptive immune systems with increased specificity. A so-called immunological memory is also established after elimination of the pathogen, to launch even stronger attack when the next invasion by the same pathogen is encountered.[11] Within the immune system, the defense structure is divided into different levels, namely, surface barriers, inflammation, complement system, and cellular barriers, among which we will only focus on the immune cells, specifically, the innate leukocytes (white blood cells). White blood cells include phagocytes, as mentioned above, that patrol the body by searching and engulfing pathogens. This type of immune cells is typically termed as neutrophils making up of a large fraction of white blood cells.[12]

We realize that neutrophils kill pathogens by direct contact that requires physical motion towards the invaders, a main characteristic to be simulated via engineered nanoparticles. Granulocytes are typical neutrophils, therefore referred to "neutrophil granulocytes" and morphologically characterized by the presence of granules in the cytoplasm. Neutrophils respond to immune-signals typically within 30 minutes, and arrive at the location rather promptly where an infection takes place (as shown in Fig. 2). The most intriguing characteristics of granulocytes are not only the ferocious eaters of micro-organisms, but also effective cancer cell killers, provided that the human body is armed with strong immunological activities (a cancer patient may have inactive granulocytes).

In conducting a mouse xerograph tumor model experiment,[13–15] Dr. Zheng Cui of Wake Forrest

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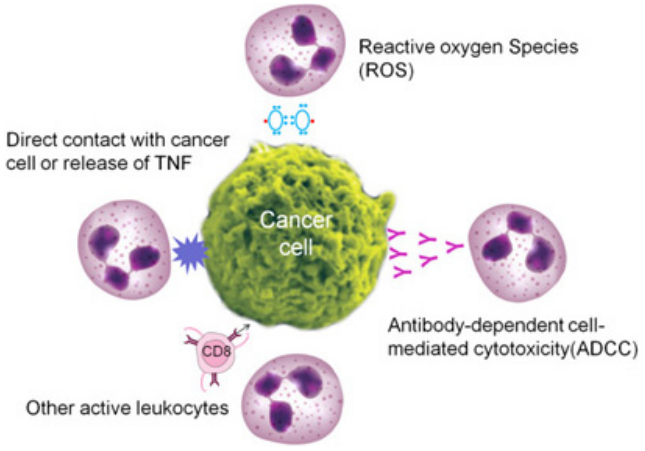

Fig. 2. Schematic diagram show the killing process of neutrophil-mediated cancer cell.

School of Medicine, found granulocytes capable of cancer cell killing, especially in some cancer-resistant mice. In a series of studies, he showed that the resistance to tumor growth was, in fact resulted from highly active granulocytes and macrophages. In the cancer-resistant mice, granulocytes surprisingly recognized the cancer cells, in a similar manner to the behavior of identifying pathogens in a normal immune response, and migrated to the vicinity of the cancer cells. Upon binding onto the cancer cell surfaces, the granulocytes consequently induced rapid cytolysis (as shown in Fig. 2).

In a video shown at https://www.youtube.com/watch?v=nJEFcNbEWQs, given by Michael Blanks and Mark Willingham of Wake Forrest University, one can see human granulocytes kill cervical cancer cells vividly. Shown in this video, a yellow arrow (appears at 0:07/0:38) points at a rather large cervical cancer cell with many surrounding granulocytes, which are relatively small with a size ratio of $\sim$5:1. These granulocytes are initially migrating in the flow direction of the fluid (to the lower right). As soon as these granulocytes enter into the vicinity of the cancer cell, they make abrupt turns and bind onto it by aggressive clustering, leading to cytolysis within minutes. A second yellow arrow appears at 0:18/0:38 pointing at another cancer cell at the lower portion of the view field. Quite similar behavior of granulocytes can be seen again with equal effectiveness (i.e. the cancer cell is destroyed within the same time frame).

The cancer cell killing mechanism has been well explained by Zheng Cui in several of his publications.[13–15] In these works, he found granulocytes effectively target and kill a fundamentally different health invader: cancer cells. Based on this finding, he developed a clinically useful *in vitro* assay for examining the "lethalness" of the cancer killer cells: granulocytes, defined as cancer killing activity (CKA).[13–15] CKA can be determined directly by co-incubating leukocytes with various tumor targets. CKA is normalized as the percentage of total target cells at selected effector to target cell ratio in comparison to no-effector-cell controls.[13–15] Using this procedure, Cui and his colleagues were able to determine CKA levels in different human control groups and found consistent variations between the healthy individuals and cancer patients. They then concluded CKA as an effective indicator on the immunological anti-cancer ability of any individual. Based on these findings, they proposed a novel therapeutic concept, namely, granulocyte infusion therapy (GIT) that utilizes active granulocytes from healthy donors for cancer patients with significantly weakened immune systems characterized by low CKA levels.[13–15]

3. Motion of Granulocytes Driven by Coulomb Force

The key point discussed in this *Technical Note* is, however, not on basic immunology for cancer cell killing by granulocytes, but rather, a new biophysical interpretation of the so-called "immune response" as seen in the video at https://www.youtube.com/watch?v=nJEFcNbEWQs. The immune-mediated cancer cell killing is often referred to "immune surveillance" or "tumor immunology".[16] As cancer cells express antigens that are not found on normal cells, the immune system may see them as foreign, and subsequently provoke immune cells to attack these transformed cells. Cancer cell killing can be described by induced cytolysis, that occurs when a cell bursts due to pressure differences across the cell membrane. Among different mediators of cytolysis in the immune system, we may only focus on granulocytes in this *Technical Note*. Similar to other Natural Killer (NK) cells, granulocytes can release perforins on the cell membrane[17] that cause a cell "leaking" by having water permeate through perforin channels. The sudden change in the membrane structure leads water to flood the cell, resulting in its burst. This is one of the major cell killing mechanisms by granulocytes that needs

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to be taken into consideration in the design and engineering of artificial cells.

However, in order to interrupt cancer cell membrane structures, these granulocytes must recognize, target, and bind onto cancer cells first. This will require them to migrate, via certain "immune response," towards to the surfaces of the cancer cells. In other words, they have to travel certain distances in the vicinity of the cancer cells. Then the questions to be asked:

(1) How do they recognize the cancer cells? and
(2) What is the driving force that moves them swiftly towards the cancer cells?

Basic biology seems to provide vague explanations about these critical questions. Before we answer these questions, let us look at another important behavior of biological cells: *bioelectricity*, which may have been overlooked in the past, particularly in the study of cell transport, cell recognition, and cell-cell interaction in physiological fluids. *Bioelectricity* can occur at different levels such as cellular, tissue, and neuronal.[18,19] At the cellular level, it is produced by the activities of ion movements across membranes, leading to establishment of cell membrane (or trans-membrane) electrical potentials.[20] For instance, the eukaryotic cells can reach a trans-membrane potential on the order of -100 mV.[20] All cancer cells exhibit negative surface charges associated with their hallmark metabolic behavior: high rate of glycolysis. The so-called "Warburg Effect" describes the cross-membrane movement of lactate, an end product of glycolysis pathway in hypoxia.[21–24] As the cancer cells constantly secrete lactic acid, upon consuming glucose, the loss of the highly mobile lactate from the cytoplasm will inevitably remove cations, such as Na^+ and H^+ at membrane surface to form lactate salts or acids, in order to complete the lactate cycle (shown in Fig. 3). In this manner, the cancer cell surfaces are left with a net of negative charges whose magnitude is regulated by glycolysis rate.[25,26]

Our previous studies have shown well-captured cancer cells (from 22 cell lines) by the positively-charged magnetic nanoparticles.[25] Most importantly, the capture rate was found to be proportional to the rate of glycolysis that was experimentally controlled and regulated by glucose uptake. Since the cancer cell surface negative charges are proportional to the glycolysis rate, more glucose consumption will result in greater secretion of lactic

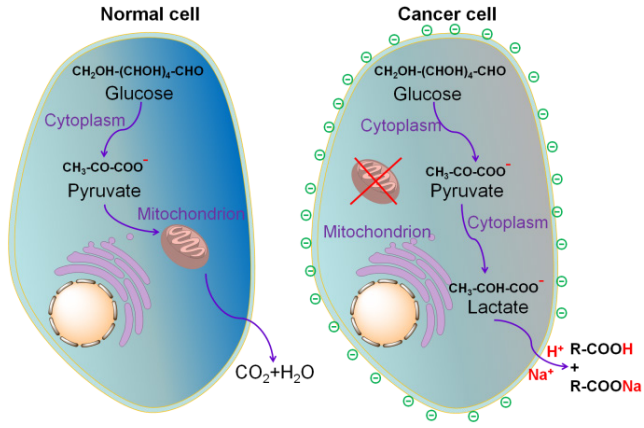
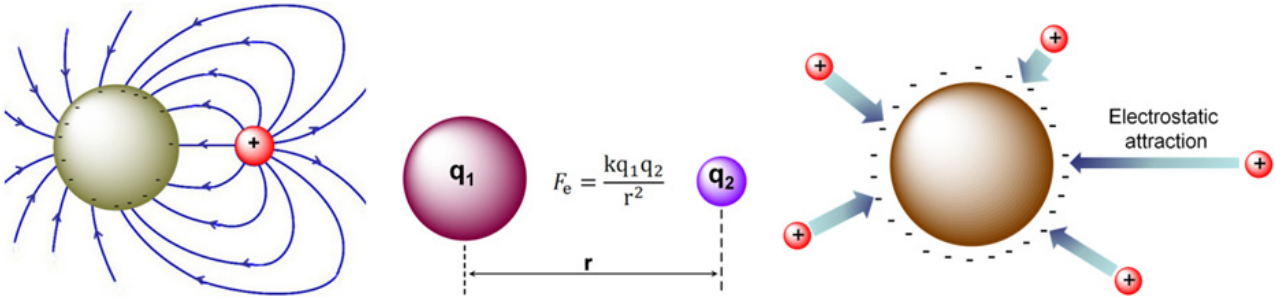


Fig. 3. Diagram showing the glycolytic-regulated cancer cell surface negative charges.

acid, thereby enhancing binding by the positively-charged nanoparticles and subsequent magnetic separation (the nanoparticles are also superparamagnetic). In the same experiment, granulocytes were found to exhibit positive charges as they were mainly captured by the negatively-charged nanoparticles. Meanwhile, three different primary non-cancerous cells (MNC, Kidney, and Liver) remained mostly nonreactive with either positive or negative nanoparticles, indicating their electrical neutrality.[25]

From the above experimental observations, we may visualize the so-called "immune response" differently from the traditional concepts and provide a new interpretation based on cell surface charges, which are mainly responsible for their fluidic behaviors, such as physical motion, cellular interaction, and short-range maneuver. Now, let us revisit the video at https://www.youtube.com/watch?v=nJEFcNbEWQs and pay attention to the motions of all granulocytes far away and around the cancer cells. Based on the fact that both cancer cells and granulocytes are oppositely charged, Coulomb force must exert on them via electrostatic interactions [Fig. 4(a)]. Figure 4(a) illustrates the electric field line distributions between a larger negatively-charged and a smaller positively-charged particle, simulating to the situation between a cancer cell (larger and negatively charged) and a granulocyte (smaller and positively charged). The consequence is that the larger (therefore heavier) cancer cell will attract those granulocytes that are in the vicinity whereby the force increases by $1/r^2$ [Fig. 4(b)].[27]

It is natural that cells of opposite charges follow the same principle that they will electrostatically



(a) (b) (c)

Fig. 4. Schematic diagrams of (a) the electric field line distributions between a larger negative sphere and a smaller positive sphere; (b) the Coulomb force between two charges, and (c) positively-charged particles being attracted to the larger one with the negative charges.

attract to each other. Since the cancer cells are normally larger (20–30 microns), they tend to "pull" the smaller granulocytes towards them, as seen from the video above that the smaller and lighter granulocytes abruptly change the directions of motion and move swiftly towards the cancer cell (the so-called "immune response" and "cell binding"). We interpret this behavior in terms of cellular electrostatic interaction and illustrate this charge-driven process in Fig. 4(c).

It should be noted that cell motion and maneuver mechanisms have been provided in the biology textbooks. In a video at https://www.youtube.com/watch?v=FD-A0MhYc7Y, one can see the conventional descriptions of cell movements by two theories, namely: Cytoskeletal Model and Membrane-Flow Model.[28,29] Although somewhat different, they basically view cells, specifically the neutrophils, as worm-like entities that move around by bodily extension, contraction, and anchoring. In the Cytoskeletal model, a cell polymerizes a sequence of actin filaments in the cell front forming the leading edge of the cell. In the back of the cell, microtubules act as a rotter that steers the direction of cellular motion. The major problem with these models is the traveling velocity of the cell being too slow in response to the signal from an invader. Furthermore, a neutrophil must respond in the range of signals by sticking to epithelium first in order to accomplish the mechanical maneuver described above (they need a "ground" to crawl) within 30 minutes (or even faster). This is particularly time consuming considering the orchestrated arrangements of all molecules in a cell. However, the linear velocity of a cell cannot be easily estimated without actual data. In our previously reported study, the charge-driven nanoparticle/cell reactions are reasonably fast. The binding of the positively-charged nanoparticles on Hela cells was found to complete within 0.1 min, far shorter than those required in the cellular endocytic reactions, especially at low temperatures. This behavior is, on the one hand, in sharp contrast to those described by the Cytoskeletal and Membrane-Flow Models, and on the other hand consistent with what is observed from the video at https://www.youtube.com/watch?v=nJEFcNbEWQs.

4. Engineering of Artificial Granulocytes

If the above hypothesis (Fig. 4) is plausible and reasonable, we may be able to design and engineer artificial granulocytes by chemical synthesis. This can be simply done by rendering the similar-sized (and mass) nanoparticles positively charged enabling them to be attracted by cancer cells in the vicinity (remember the $1/r^2$ law so they have to be close enough to be electrostatically effective). To conform this hypothesis, we simulate the biological process of the "immune response" by assembly of multifunctional nanoparticles with required electric properties (charge). A spherical nanoparticle of 100 nm diameter can be constructed by a variety of materials including both polymeric and inorganic materials, provided that they assume charged and monodispersed "granular" characteristics [Fig. 5(a)]. The surface charges can be decoratively attached on the nanoparticle surfaces by conjugation with chemical functional groups, typically, polyethylenimine (PEI) molecules for exhibiting the positive charges [Fig. 5(b)]. In our previous studies, the nanoparticles can be readily charged, by surface functionalization,

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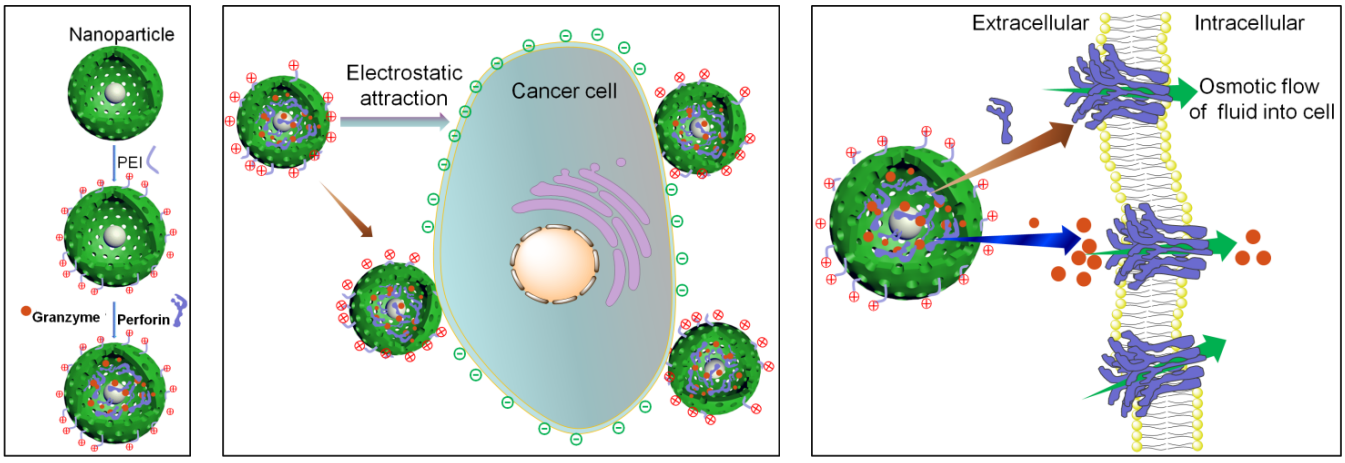

(a) (b) (c)

Fig. 5. Schematic diagram showing the concept of artificially engineered granulocytes.

with electrical voltage in the range of 20–40 mV, that is sufficient for cell targeting, binding, and capturing. As shown in Fig. 5(b), similar to the video at https://www.youtube.com/watch?v=nJEFcNbEWQs, the positively-charged nanoparticles can be electrostatically attracted to the negatively-charged cancer cell, in a similar fashion that granulocytes bind onto the cancer cells. Upon on binding, the engineered granulocytes are able to perform the same tasks in cell killing [Fig. 5(c)] if the perforin release mechanism can be installed.

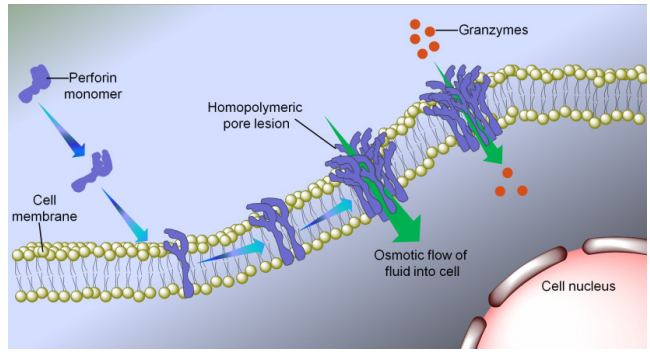

Fig. 6. Schematic diagram of perferin-induced cytolysis.

5. Cytolysis by Artificial Granulocytes

By simulating some of the biological characteristics, such as surface charges, the engineered artificial cells may function as neutrophils that target and bind cancer cells (or pathogens since they too are negatively charged) in the similar manner as granulocytes. However, this is only to enable them maneuver in the blood stream and dock the cancer cells. To induce cytolysis, certain cancer cell killing mechanism will have to be established in the artificial cells. Perforin is a cytolytic protein that binds onto the cancer cell membrane, and forms pores on it.[17]

Once the channels are formed on the cell membrane, cell death will result from either partial pressure induced burst, or diffusion of granzymes into the target cell (as shown in Fig. 6). It is possible to engineer the artificial cells with the perferin release mechanism via chemical synthesis. It should be noted that cell engineering is specifically intended for simulating granulocytes in this *Technical Note* for their positively-charged nature having been already verified in our previous works.[25,26] The biological properties of other cells are to be investigated and simulated in the future studies for different purposes.

Based on the understanding of granulocytes, we have provided two major functions of an artificial neutrophil:

(1) Surface charge for cell maneuver, and
(2) Surface perferin for cell killing.

Based on well-established chemical synthesis and nanotechnology, it is highly feasible and worthy to undertake this experimental investigation to confirm the above hypothesis. It should be noted that there have been a variety of cancer cell killing mechanisms developed, such as drug/gene delivery

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and photothemal ablation.[3–7] But for the purpose of "cell engineering," it is required to simulate a cell based on all its characteristics, while having equal or even better effectiveness. For this reason, the simulation of granulocytes, surface perferin release will be necessary, in order to develop a "neutrophil-like" cell. Furthermore, the artificial cell is highly preferred to be biocompatible and biodegradable for the obvious reasons. It is also intended to simulate other biological systems (such as T-cells), based on fundamental understanding of their biological properties and mechanisms.

6. Conclusions

Granulocytes are lethal cancer cell killers as shown in several previous studies. In particular, it is their positively-charged nature that makes them responsible for aggressive binding onto the negatively-charged cancer cells. Release of perferin is a common behavior of neutrophils in the process of cell killing. Based on the understanding of neutrophils, especially their electrical properties, artificial granulocytes can be engineered by chemical means. While the positive charges can be decorated on spherical nanoparticles, perferin is to be released with surface engineered functionalities. Using this approach, many more types of cells can be designed and engineered, provided that the basic cell biological properties are well understood and simulated.

As has been known, neutrophils are capable of recognizing cancer cells and pathogens. Yet the recognition mechanism can be well and simply explained by cell *bioelectricity*. In fact, both cancer cells and pathogens are known to be negatively charged, and therefore, the positively-charged granulocytes are able to recognize them via electrostatic interaction, and at the same time avoid the normal cells that are more or less neutral.

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