Core-Shell Silver/Polymeric Nanoparticles-Based Combinatorial Therapy against Breast Cancer

In-vitro

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The current study aimed at preparing AgNPs and three different core-shell silver/polymeric NPs composed of Ag core and three different polymeric shells: polyvinyl alcohol (PVA), polyethylene glycol (PEG) and polyvinylpyrrolidone (PVP). Thereafter, the core/shell NPs were loaded with a chemotherapeutic agent doxorubicin (DOX). Finally, the cytotoxic effects of the different core-shell Ag/polymeric NPs-based combinatorial therapeutics were tested in-vitro against breast cancer (MCF-7) and human fibroblast (1BR hTERT) cell lines. AgNPs, Ag/PVA and Ag/PVP NPs were more cytotoxic to MCF-7 cells than normal fibroblasts, as well as DOX-Ag, DOX-Ag/PVA, DOX-Ag/PEG and DOX-Ag/PVP nanocarriers (NCs). Notably, low dosage of core-shell DOX-loaded Ag/polymeric nanocarriers (NCs) exhibited a synergic anticancer activity, with DOX-Ag/PVP being the most cytotoxic. We believe that the prepared NPs-based combinatorial therapy showed a significant enhanced cytotoxic effect against breast cancer cells. Future studies on NPs-based combinatorial therapy may aid in formulating a novel and more effective cancer therapeutics.

Breast cancer is the most common type of cancer among women worldwide¹,². DOX is an FDA-approved chemotherapeutic agent frequently used in the treatment of various cancers including breast cancer. DOX is an anthracycline drug that inhibits topoisomerase-II-mediated DNA repair and leads to cell apoptosis³. Despite the potent anticancer action of DOX, it mediates cardiotoxicity. It was reported that DOX cumulative dose was the only confirmed risk factor for DOX-mediated cardiotoxicity. Furthermore, drug-induced cancer resistance is another obstacle that limits DOX clinical use⁴. A current approach used to overcome the resistance problem is the utilization of two or more chemotherapeutics⁵. Despite the synergism mediated by such combination chemotherapies, clinical studies showed that pharmacokinetic interactions of such combination chemotherapies induced severe systemic side effects such as cardiotoxicity and bone marrow suppression⁶–⁷. Consequently, there is an urgent need for the development of novel strategies to treat cancer. One strategy is to modify a well-studied chemotherapeutic agent, e.g. DOX- that would ideally: (i) target and kill cancer cells selectively, (ii) have an improved efficacy/toxicity balance, (iii) have an enhanced therapeutic index and (iv) have an improved pharmacokinetics profile; when compared to the original non-modified drug.

Nanotherapeutics usage in drug delivery applications has recently increased because of their desirable therapeutic characteristics e.g. prolonged systemic circulation and targeted drug delivery⁸–⁹. The previous properties are particularly advantageous for cancer therapeutics because they would result in an improved chemotherapeutic efficacy and would minimize the systemic toxicity⁸–¹⁰. The advent of nanotechnology allowed the emergence of several formulation techniques of NPs-based combinatorial therapy. These techniques include multiple chemotherapeutics using a single NC in order to overcome the drug-induced cancer resistance¹¹. Recently, another approach similarly employed NPs-based combinatorial therapy, utilizing NPs with anti-cancer activity in combination with a chemotherapeutic agent. Ostad et al. used this approach and reported that administrating a low dose of tamoxifen following AgNPs to breast cancer cells and tamoxifen-resistant cancer cells induced...
a synergistic anticancer effect against both cancer cell lines. Several studies reported that the cytotoxicity of core-shell Ag/PVP NPs was attributed to their ability to bypass the cell membrane via endocytosis and to localize into the lysosomes. Here, the NPs are exposed to an acidic environment that triggers the release of silver ions, eventually inducing the generation of reactive oxygen species (ROS). The generated ROS and AgNPs escape from the lysosomes and then they disrupt the mitochondria. This event leads to the generation of more ROS, ultimately leading to DNA damage and cell death. In addition, AgNPs were reported to possess potent anti-angiogenic effects via inhibition of vascular endothelial growth factor (VEGF)-induced angiogenesis both in-vitro and in-vivo. In addition, Kwon et al. showed that surfactant-coated AgNPs are hemo-compatible with human erythrocytes, and their conclusion supports the idea of intravenous administration of AgNPs as well as their potential use for cancer therapy.

This study aimed at formulating combinatorial nanotherapeutics by conjugating DOX onto core-shell Ag/polymeric NPs. The NPs were composed of Ag as the core and three FDA-approved polymers (PVA, PEG and PVP) as the shell (Fig. 1). The drug loading and in-vitro drug release of DOX-NCs were monitored and their cytotoxic effects on MCF-7 cells and 1BR hTERT cells were assessed. Our results demonstrate that core-shell Ag/polymeric NPs and DOX-NCs had higher efficacy in killing MCF-7 cells -in comparison to their unloaded counterparts. Our results imply the high potential of the use of the synthesized DOX-loaded Ag/polymer NCs as nanotherapeutics for breast cancer treatment in the future.

Results

Synthesis and Characterization of AgNPs and core-shell NPs. The size and morphology of the prepared AgNPs and core-shell Ag/polymeric NPs were characterized by TEM, SEM, and UV-visible spectroscopy. TEM and SEM images showed that the prepared AgNPs and core-shell Ag/polymeric NPs were spherical, mono-dispersed, and well-dispersed (Fig. 2). The size and zeta potential of AgNPs and core-shell Ag/polymeric NPs (Table 1) were measured using the Zetasizer and were found to be in the range of 15–28 nm. The zeta potential values of AgNPs, Ag/PVA NPs, Ag/PEG NPs and Ag/PVP NPs were $-12.43 \pm 1.20$ mV, $-0.30 \pm 0.50$ mV, $-2.35 \pm 1.76$ mV and $-12.4 \pm 1.20$ mV respectively. Although the zeta potential of core-shell NPs demonstrated relatively low values, these NPs are stable due to the presence of a large molecular weight stabilizer that acts mainly by steric stabilization. This is based on the fact that the adsorbed polymer layer shifts the plane of shear to a further distance from the particle system and thus results in a reduction in the value of the measured zeta potential. The UV-Vis spectra of AgNPs, Ag/PVA NPs and Ag/PEG NPs (Fig. 3A–D) showed a sharp Plasmon absorption peak at ~400 nm, which is the characteristic peak of spherical, mono-dispersed and well-dispersed AgNPs. However, the UV-Vis spectrum of Ag/PVP NPs (Fig. 3D) showed a sharp peak at ~420 nm. Previous studies demonstrated that spherical and mono-dispersed Ag/PVP NPs display a SPR band at ~412–437 nm. The FT-IR spectra also confirmed the formation of AgNPs and core-shell Ag/polymeric (PVA, PEG, and PVP) NPs (Supplementary Figs S1–S4).

Synthesis and Characterization of DOX-NCs. Following the synthesis of AgNPs and core-shell Ag/polymeric NPs, each individual type of NP was loaded with DOX. The drug loading efficiency was determined based on the DOX content in the supernatant. The drug loading efficiency percentages (Table 1) were determined to be: 58.3%, 54.9%, 56.5% and 62.5% for AgNPs, core-shell Ag/PVA NPs, core-shell Ag/PEG NPs and Ag/PVP NPs, respectively. The conjugation between DOX and NPs was detected using, TEM, SEM, and UV-Vis spectra (Fig. 3). The UV-Vis spectra (Fig. 3I–L) indicated that the binding between DOX and NPs resulted in a red shift of the Plasmon absorption band of loaded NCs from 400 to ~500 nm. The size and zeta potential of DOX-NCs (Table 1) were also measured and
the results indicated that the size of NCs were not significantly increased as compared to their unloaded counterparts. The zeta potential values of the DOX-NCs (Table 1) were shifted to more negative values compared to their unloaded counterparts, and thus confirming the stability of the synthesized DOX-NCs. Previous studies demonstrated that the negatively charged NCs were beneficial for biomedical applications because they were slowly eliminated from the blood stream and had a lower cytotoxicity as compared to positively charged NCs28,29.

**In-vitro drug release.** Since the release behavior of DOX-NCs at the desired site is of a great importance for formulating an ideal cancer-targeted drug delivery system, *in-vitro* release studies were performed. For this purpose, two different pH values were tested: pH 7.4, which mimics the pH of the blood stream and pH 5, which mimics the pH of the endosomes within cancer cells. *In-vitro* release study results (Fig. 4A,B) showed that DOX-AgNCs, DOX-Ag/PVA NCs, DOX-Ag/PEG NCs, and DOX-Ag/PVP NCs released 96.6%, 97.4%, 98% and 96.4% of DOX at pH 5. While at pH 7.4, the release percentages of DOX were 73.4%, 54.3%, 59.8% and 68.5% over the period of 6 hrs. On the other hand, free DOX solution was also used as a control and it was found that free DOX released 97.4% of DOX at pH 5, and 67.7% at pH 7.4 over the period of 4 hrs.

**In-vitro Cytotoxicity assay.** Effect of AgNPs and Core-shell Ag/polymeric on MCF-7 cells and 1BR hTERT cells. In order to assess the cytotoxic effect of AgNPs and core-shell Ag/polymeric NPs, MCF-7 and 1BR hTERT cells were exposed separately to different concentrations of (0, 10, 20, 50, and 100 μg/mL) NPs for 48 hrs. AgNPs and core-shell Ag/polymeric (PVA, PEG and PVP) NPs decreased the cell viability of MCF-7 cells and 1BR hTERT cells (Fig. 5A–D) in a dose-dependent manner. The inhibitory concentration (IC50) was estimated to be 48 μg/mL for AgNPs, 42 μg/mL for Ag/PVP NPs and greater than 100 μg/mL for both Ag/PVA NPs and Ag/PEG NPs on MCF-7 cells. The IC50 of NPs in 1BR hTERT cells was estimated to be 100 μg/mL for AgNPs, Ag/PVA NPs, and Ag/PEG NPs, while the IC50 of Ag/PVP NPs was 50 μg/mL. The Ag/PVA and Ag/PVP NPs were more

Table 1. Composition, Size, Zeta potential, and drug loading of prepared NPs and DOX NCs.
cytotoxic against cancer cells at the high concentration of 100 μg/mL, with Ag/PVP NPs being more cytotoxic against MCF-7 cancer cells (Fig. 5A–D).

**Effect of DOX-core-shell Ag/polymeric NPs on MCF-7 cells and 1BR hTERT cells.** To investigate the cytotoxic effect of NPs-based combinatorial therapy, different concentrations of free DOX (2, 4, 8, 10, and 12 μg/mL) were tested on MCF-7 and 1BR hTERT cells, and cell viability was measured after 48 hrs. The IC₅₀ of free DOX on MCF-7 cells was determined to be 3.7 μg/mL (Supplementary Fig. S5). Based on the IC₅₀ of free DOX, lower DOX-NCs concentrations than the calculated IC₅₀ of free DOX were selected (0.1, 0.2, and 1 μg/mL DOX) in
order to assess whether the combination between DOX and NPs would induce synergism or not. The estimated IC50 values of DOX-NCs against both MCF-7 cells and 1BR hTERT cells, together with the individual concentrations of DOX and Ag in each DOX-NC—which lead to 50% cytotoxicity of both cell lines—are presented in Table 2.

The estimated IC50 values (Table 2) of DOX-AgNCs, DOX-Ag/PEG NCs, and DOX-Ag/PVP NCs against MCF-7 cells were 1.00–11.23 μg/mL, 0.14–3.00 μg/mL, and 0.10–3.50 μg/mL, respectively (Fig. 6A–D). On the other hand, the estimated IC50 values (Table 2) of DOX-NCs against 1BR hTERT cells were 1.00–11.23 μg/mL for DOX-AgNCs, DOX-Ag/PEG NCs, and DOX-Ag/PVP NCs, while the estimated

Figure 4. In-vitro release of free DOX, and DOX-NCs in Tris-HCl buffer pH 5 (A) and PBS pH 7.4 (B).

Figure 5. The percentage of viable MCF-7 cells (red) and 1BR hTERT cells (blue) as determined by the MTT assay following 48 hrs incubation with concentrations of 0, 10, 20, 50 and 100 μg/ml of: (A) AgNPs, (B) Ag/PVA NPs, (C) Ag/PEG NPs and (D) Ag/PVP NPs. The data are presented as means of at least three independent experiments (mean ± SD). P values were calculated for each concentration, and denoted if found to be significantly different between the two cell lines (*P < 0.05, **P < 0.01 and ***P < 0.001).
IC$_{50}$ value of DOX-Ag/PVA NCs was 0.60–9.00 μg/mL (Fig. 6A–D). All DOX-loaded core-shell Ag/polymeric NCs were found to be more cytotoxic against cancer cells versus normal cells. Notably, the Dox-Ag/PVP combination was more cytotoxic than all three and was more cytotoxic on cancer cells.

Discussion
In other studies DOX was loaded to different carriers such as liposomes, polymeric NPs, carbon nanotubes, however, few studies focused on formulating a combination therapy based on using DOX and AgNPs. In this study, DOX was loaded to AgNPs and core-shell Ag/polymeric NPs. DOX loading was confirmed by UV-visible spectroscopy. The UV-Vis spectra of DOX-NCs (Fig. 3I–L) showed a red shift - consistent with previously published data$^{30,31}$, which probably resulted from a change in pH due to the interaction of the NPs with DOX –which in itself is of acidic nature. The red shift of UV-Vis spectra was also attributed to the binding between DOX and AgNPs, which resulted in the decrease in inter-particle distance of NPs. Though the red shift of UV-Vis spectra and low values of Zeta potential both suggest that there are some NPs aggregations, however, the TEM and SEM images of DOX-NCs (Fig. 3) confirmed that the DOX-NCs remained well-dispersed. A similar observation on the well-dispersity of the NCs after their binding with DOX has also been reported by Kumar et al.$^{30}$. Therefore, by combining the results obtained from the UV-Vis spectra, the TEM images, as well as the SEM images of unloaded-NPs and DOX-NCs, it can be inferred that DOX and NPs were successfully binding to each other while maintaining the well-dispersity of the NCs.

The in-vitro release studies (Fig. 4A,B) also demonstrated that free DOX was released faster than DOX-NCs at both of the tested pH values. The delay of DOX release from DOX-NCs was due to the binding of DOX with

### Table 2. Estimated IC$_{50}$ values of DOX-NCs against both MCF-7 cells and 1BR hTERT cells.

| Formulae       | IC$_{50}$ | MCF-7 cells | 1BR hTERT cells |
|----------------|-----------|-------------|-----------------|
| DOX-AgNCs      | 1.00 11.23 | 1.00 11.23  |
| DOX-Ag/PVA NCs | 0.19 3.40  | 0.60 9.00   |
| DOX-Ag/PEG NCs | 0.14 3.00  | 1.00 11.23  |
| DOX-Ag/PVP NCs | 0.10 3.50  | 1.00 11.23  |

IC$_{50}$ value of DOX-Ag/PVA NCs was 0.60–9.00 μg/mL (Fig. 6A–D). All DOX-loaded core-shell Ag/polymeric NCs were found to be more cytotoxic against cancer cells versus normal cells. Notably, the Dox-Ag/PVP combination was more cytotoxic than all three and was more cytotoxic on cancer cells.

**Figure 6.** The percentage of viable MCF-7 cells (red) and 1BR hTERT cells (blue) as determined by the MTT assay following 48 hrs incubation with concentrations of 0, 0.1, 0.2 and 1 μg/ml (concentrations are referring to DOX concentration) of: (A) DOX-Ag NCs, (B) DOX-Ag/PVA NCs (C) DOX-Ag/PEG NCs, and (D) DOX-Ag/PVP NCs. The data are presented as means of at least three independent experiments (mean ± SD). P values were calculated for each concentration between the two cell lines, and denoted if found to be significant (*P < 0.05, **P < 0.01 and ***P < 0.001).
the different NPs, which accordingly improved the release profile of DOX and prolonged its half-life compared to free DOX. Moreover, the results confirmed that both DOX-NCs and free DOX exhibited faster release in pH 5, which mimics the pH of endosomes within cancer cells, when compared to their release in pH 7.4. The fast release of free DOX was based on the fact that protonated DOX has a higher solubility. However, the fast release of DOX from DOX-NCs was due to weakened interaction between DOX and NCs at acidic pH. This is due to the protonation of DOX amino groups, which leads to DOX detachment from the NPs. The pH-sensitivity property of DOX-AgNCs complexes seems to be advantageous for cancer-targeted drug delivery because the acidic microenvironment of cancer cells facilitates active drug release from NCs, increases drug bioavailability to cancer cells, and leads to high therapeutic efficacy compared to normal cells.

The cytotoxicity of unloaded AgNPs and core-shell Ag/polymeric NPs were examined on MCF-7 cells and IBR hTERT cells by the MTT assay. AgNPs and core-shell Ag/polymeric NPs – except Ag/PEG NPs – showed higher cytotoxicity on cancer MCF-7 cells compared to normal IBR hTERT cells (Figs 5 and 6). These results imply that AgNPs particularly coated with PVP, and to a lesser extent coated with PVA, are specifically cytotoxic to MCF-7 cancer cells, when compared to normal IBR hTERT cells. This is in contrast to the PEG coating, which resulted in more cytotoxicity to normal cells. Based on previous studies, the cytotoxic effect of AgNPs was ascribed to their ability to dissolve and release Ag⁺ ions, which have a great potential to translocate to both the mitochondria and nucleus, thereby triggering the generation of ROS and mediating oxidative stress. The oxidation stress causes a series of cellular events including the reduction of glutathione (GSH) and superoxide dismutase (SOD) levels as well as increasing lipid peroxidation, which finally lead to DNA damage and cancer cell death. The difference in the estimated IC₅₀ values among the NPs is probably attributed to the different surface coating among NPs. Previous studies documented that the surface coating of AgNPs controls their dissolution and influences their cytotoxicity. In concordance, this study confirmed that the surface coating of NPs directly influences their cytotoxicity.

Preparation of AgNPs and core-shell Ag/polymeric NPs were synthesized by polyol process. All preparation methods were described in the supplementary information.

DOX and core-shell Ag/polymeric (PV A, PEG, and PVP) NPs were successfully synthesized, loaded with DOX, and the in-vitro drug release of each individual type of DOX-NCs was investigated. Moreover, an individual unloaded-NP, free DOX and DOX-NC were tested for in-vitro cytotoxicity on MCF-7 cells and IBR hTERT cells. In-vitro MTT assay results demonstrated that core-shell DOX-Ag/polymeric NCs at much lower doses- showed a synergic cytotoxic effect towards MCF-7 cells, and a lower cytotoxic effect on normal IBR hTERT cells. Finally, to complement and confirm the synergism and overall efficacy of DOX-Ag/polymeric NCs, several studies such as in-vitro and in-vivo toxicity studies and in-vivo anti-tumor activity on cancer cells and normal cells are recommended. These further studies could progress the proposed NPs-based combinational therapeutic to formulate a novel targeted cancer therapy that could be used in clinical trials; as it can potentially eradicate cancer cells selectively and effectively while minimizing the adverse side effects.

Conclusion
Mono-dispersed spherical AgNPs and core-shell Ag/polymeric (PVA, PEG, and PVP) NPs were successfully synthesized with DOX, and the in-vitro drug release of each individual type of DOX-NCs was investigated. Moreover, an individual unloaded-NP, free DOX and DOX-NC were tested for in-vitro cytotoxicity on MCF-7 cells and IBR hTERT cells. In-vitro MTT assay results demonstrated that core-shell DOX-Ag/polymeric NCs at much lower doses- showed a synergic cytotoxic effect towards MCF-7 cells, and a lower cytotoxic effect on normal IBR hTERT cells. Finally, to complement and confirm the synergism and overall efficacy of DOX-Ag/polymeric NCs, several studies such as in-vitro and in-vivo toxicity studies and in-vivo anti-tumor activity on cancer cells and normal cells are recommended. These further studies could progress the proposed NPs-based combinational therapeutic to formulate a novel targeted cancer therapy that could be used in clinical trials; as it can potentially eradicate cancer cells selectively and effectively while minimizing the adverse side effects.

Methods
Preparation of AgNPs and core-shell NPs. AgNPs, core-shell Ag/PVA NPs and Ag/PEG NPs were prepared by chemical reduction method with some modification as reported previously. Core-shell Ag/PVP NPs were synthesized by polyol process. All preparation methods were described in the supplementary information.
Synthesis of DOX-AgNCs. Briefly, 1 mL aqueous solution of DOX (0.2 mg/mL) was mixed with an aqueous solution (2 mg/mL) of each individual type of AgNPs at pH 7.4. Each mixture was shaken in a rotary shaker at 37 °C for 24 hrs in dark conditions. Then, the mixtures were centrifuged for 15 min and the supernatants were used to determine the amount of free DOX by UV-Vis spectroscopy at 480 nm30. The drug loading efficiency was calculated as follows30:

\[
\text{Drug Loading Efficiency} \, (\%) = \frac{\text{(Initial amount of DOX)} - \{\text{Supernatant free amount of DOX}\}}{\text{(Initial amount of DOX)}} \times 100
\]

\[\text{In-vitro drug release.}\] 1 mL of individual DOX-NC was dispersed in de-ionized water and then transferred into a dialysis bag (cut off molecular weight 12,000–14,000 g/mol, Serva, Germany) containing 50 mL PBS buffer (pH 7.4) and Tris-HCl buffer (pH 5) respectively, with the temperature maintained at 37 °C. At fixed time intervals, 1 mL of the medium from each dialysis bag was withdrawn and subsequently replaced with fresh buffer to maintain the sink conditions30. The amount of released DOX was determined by UV-Vis spectroscopy at 480 nm. The cumulative percentage of drug release was calculated as follows30:

\[
\text{Cumulative drug release} \, (\%) = \frac{\{\text{Amount of DOX in the release medium}\}}{\text{(Initial amount of DOX loaded onto NPs)}} \times 100
\]

Cytotoxicity assay. The effects of different NPs were tested on two distinct, yet conventional cell lines; wild-type human telomerase reverse transcriptase immortalized hTERT cells-1BR skin fibroblast (1BR hTERT cells) and the human breast adenocarcinoma cell line (MCF-7)42, 1BR hTERT cells, were a gift of Dr. Andreas Kakarougkas (University of Sussex), were used as control cells; as they are non–cancerous –normal- immortalized human skin fibroblasts42-45. The cytotoxicity experiments aimed to assess and compare the cytotoxicity of the different studied NPs on breast cancer cells –MCF-7 cells- and normal fibroblasts -1BR hTERT cells. The cytotoxic effect of the aforementioned concentrations of DOX, AgNPs, core-shell Ag/polymeric NPs, and the DOX-NCs on MCF-7 cells and 1BR hTERT fibroblasts were conducted by the 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay27,32. The absorbance (A) was measured using FLUOstar OPTIMA microplate reader (BMG LabTech, Germany) at 595 nm. The percentage of cell viability was calculated as follows32,34:

\[
\text{Cell viability} \, (\%) = \frac{[A \, \text{(sample)} - A \, \text{(blank)}]/A \, \text{(control)}}{A \, \text{(control)}} \times 100
\]

For the IC50 values (Table 2), the Dox concentrations were calculated from the cytotoxicity assay data using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com. For the corresponding Ag IC50 values, they were calculated in relevance to Ag and by the DOX loading efficiency (equation 1)27.

Statistical analysis. All the cell viability percentage values (Figs 5 and 6) were analyzed by Tukey’s HSD statistical test one-way ANOVA pair-wise comparisons by using version 2.14.1.

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