Selective killing of cells triggered by their mRNA signature in the presence of smart nanoparticles

Amelie Heuer-Jungemann,[a] Afaf H. El-Sagheer,[c,d] Peter M. Lackie,[e] Tom Brown[c] and Antonios G. Kanaras[a,b*]

The design of nanoparticles that can selectively perform multiple roles is of utmost importance for the development of the next generation of nanoparticulate drug delivery systems. So far most research studies are focused on the customization of nanoparticulate carriers to maximize their drug loading, enhance their optical signature for tracking in cells or provide photo-responsive effects for therapeutic purposes. However, a vital requirement of the new generation of drug carriers must be the ability to deliver their payload selectively only to cells of interest rather than the majority of various cells in the vicinity. Here we show for the first time a new design of nanoparticulate drug carriers that can specifically distinguish different cell types based on their mRNA signature. These nanoparticles sense and efficiently kill model tumour cells by the delivery of an anti-cancer drug but retain their payload in cells lacking the specific mRNA target.

Despite their great potential, applications of nucleic acids in live cell are limited due to their inability to transfect cells without the aid of co-carriers and their inherent susceptibility to intracellular degradation. As a consequence different strategies have been developed for cellular nucleic acid delivery (e.g. conjugation to, or encapsulation within cationic transfection agents,[1] liposomes[2] or nanoparticles[3] as well as introduction of non-natural modifications[4]). Recently, Mirkin and co-workers reported that gold nanoparticles coated with a dense shell of oligonucleotides are ideal candidates for biomedical applications.[5] Owing to the 3-D arrangement of the oligonucleotides on the nanoparticle core, the so-called spherical nucleic acids (SNAs)[6] have shown excellent biocompatibility, bio-stability and uptake properties by a plethora of different cell types, without the requirement for additional carriers.[7, 8] Based on the SNA design, Mirkin and co-workers introduced highly stable and specific sensors for the detection of biomolecules, other small molecules or specific ions.[5, 9] To date, despite their great success as live cell imaging. agents, there have been limited reports of further developing these probes to explore their full potential in therapy.[10]

In this work we present for the first time the development of gold-nucleic acid nanoparticulate systems, which have the information encoded into their ligand substrate to selectively sense mRNA and simultaneously deliver a drug payload to live cells (see Scheme 1).

These systems were specifically designed to detect and interfere with epithelial to mesenchymal transition (EMT), a process often associated with tumour metastasis.[11] This process involves the transition of stationary, non-motile epithelial cells into invasive and highly motile mesenchymal cells. During EMT, epithelial cells undergo extensive changes in mRNA and protein expression profiles, allowing them to adopt mesenchymal characteristics.[12] These include increased motility, enhanced elasticity and invasive behaviour.[13] An mRNA and corresponding protein strongly up-regulated in EMT is the intermediate filament protein and mesenchymal cell marker Vimentin.[14-17] In contrast, epithelial cell markers Desmocollin and Keratin 8 are strongly down-regulated. [18] Accordingly, we selected these three important biomarkers as suitable model targets (see Fig.S1 for immunofluorescent labelling of relevant proteins in both model cell lines). Our gold-nucleic acid probes are able to detect the presence of these specific mRNA markers and selectively release the anticancer drug Doxorubicin (DOX), resulting in a highly efficient cellular death. Many anti-cancer drugs to date suffer from severe side-effects due to a lack of specificity. Thus, the ability to specifically detect metastatic cancer cells and efficiently deliver a therapeutic drug at the same time, in a controlled and highly targeted approach, is a significant advance.

Scheme 1 illustrates the design of the nanoparticle probes. They consist of four important parts: 1) a gold nanoparticle core 2) fluorophore-tagged oligonucleotides (sense strand)

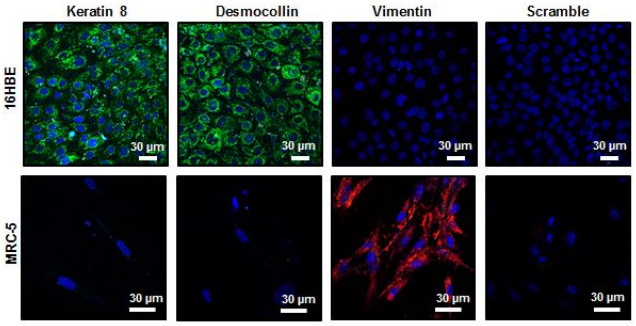


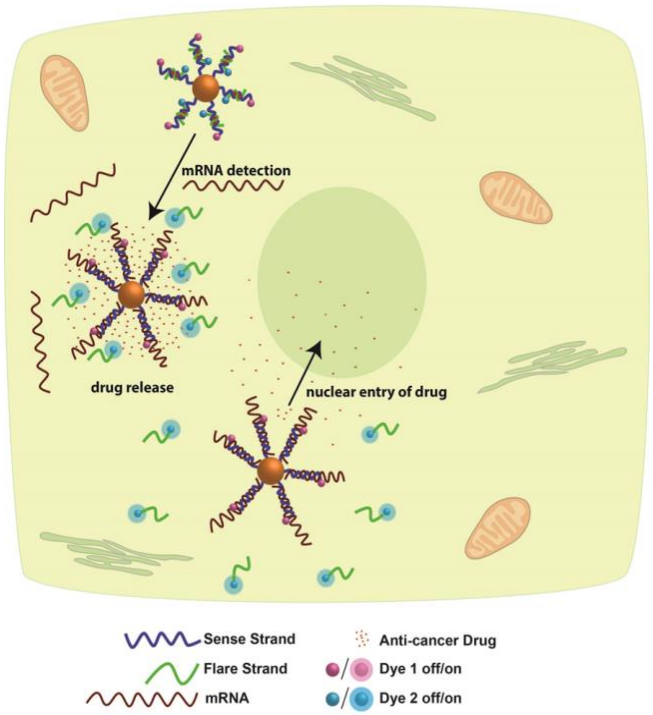
Figure 1. Live cell confocal microscopy images of cells incubated with different specific nano-probes. Epithelial (16 HBE) and mesenchymal (MRC-5) cell lines were incubated for a period of 18 h with Keratin 8, Desmocollin or Vimentin nano-probes prior to visualisation of fluorescence by live cell confocal microscopy. Colour guide: Keratin 8 and Desmocollin: Flare strand – green, sense strand – red. Vimentin and Scramble: Flare strand – red, sense strand – green, nuclear counter stain - blue.

attached to the nanoparticles, 3) fluorophore-tagged oligonucleotides (flare strand) that are released from the

[a] Physics and Astronomy, Faculty of Physical Sciences and Engineering
[b] Institute of Life Sciences, University of Southampton, Southampton, SO171BJ, UK. E-mail: A.Kanaras@soton.ac.uk
[c] Department of Chemistry, University of Oxford, Chemistry Research Laboratory, 12 Mansfield Road, Oxford, OX1 3TA, UK.
[d] Chemistry Branch, Department of Science and Mathematics, Faculty of Petroleum and Mining Engineering, Suez University, Suez 43721, Egypt.
[e] Clinical and Experimental Sciences, Faculty of Medicine, University of Southampton, Southampton, SO16 6YD, UK.

nanoparticles in the presence of a specific target and 4) a DNA intercalating anti-cancer drug.

The close proximity of the fluorophores to the gold nanoparticle core results in fluorescence emission quenching. In the presence of the specific mRNA target, the flare strand is displaced by competitive hybridisation and its fluorescence signal is restored as it diffuses away from the gold nanoparticle



Scheme 1. Schematic illustration of the nanoparticulate drug carrier system. Gold nanoparticles were functionalised with fluorophore-tagged oligonucleotides (the sense strand) complementary to the mRNA sequences of Vimentin, Desmocollin or Keratin 8. Hybridised to the sense strand is a short fluorophore-tagged oligonucleotide strand (the flare strand). Due to the close proximity of the dyes to the gold nanoparticle, their fluorescence is quenched. The anti-cancer drug doxorubicin is intercalated between the sense-flare duplex. In the presence of the specific mRNA, the flare strand is released through competitive hybridisation, thus restoring its fluorescence signal whilst simultaneously releases the drug. Upon nuclear entry, doxorubicin interferes with nuclear DNA, resulting in cell death.

core. Simultaneously the drug is released from the nanoparticles and enters the cell nucleus, where it interferes with genomic DNA resulting in apoptosis. [19] The incorporation of a fluorescent dye on the sense strands represents an important alteration to previous designs. It serves as a self-reporting mechanism ensuring that any fluorescent signal observed from the flare strand is due to the detection of the target mRNA rather than intracellular DNA degradation. In the case of intracellular degradation or the displacement of the sense strand from the gold nanoparticle, fluorescent signatures of both dyes would be observable.

Prior to live cell applications, it was imperative to ensure that the probes exhibit excellent bio-stability and specificity. The two main factors affecting intracellular stability are degradation by nucleases and the displacement of oligonucleotides from the AuNP surface by competitive conjugation of glutathione through its cysteine side chain – both would result in a false signal. Through various assays (Fig. S4 and S5), we determined that the nanoparticle probes used here, were not significantly affected by the cytosolic DNase I, lysosomal DNase II or glutathione in a time frame of 18 h within the conditions tested. As discussed in previous reports, this is most likely due to the arrangement of oligonucleotides on the AuNP core as well as the high local cation concentration between neighbouring DNA strands, both of which are detrimental to the function of nuclease enzymes.[20] We furthermore elucidated that in addition to their exceptional stability, the probes displayed excellent target specificity, with minimal recognition of targets containing up to two DNA mismatches (Fig. S3).

Subsequent live cell experiments were conducted, investigating the uptake properties and specificity of probes for the detection of Vimentin, Keratin 8 and Desmocollin in both epithelial and mesenchymal cells (Fig. 1). Cells were analysed by confocal microscopy to visualise intracellular fluorescence. The nanoparticle probes displayed a high fluorescent signal in the presence of their specific target without any detectable signal from the anchored sense strand. Therefore intracellular degradation can be excluded as a cause of the fluorescent signals observed. Whilst Desmocollin and Keratin 8 are only expressed in the epithelial cells (16HBE), Vimentin is exclusively expressed in the mesenchymal cells (MRC-5). Accordingly, both the Desmocollin and Keratin 8 nano-probes displayed activity in the epithelial cells only, while the Vimentin nano-probes revealed a fluorescent signal solely in the mesenchymal cells. Nano-probe specificity was further confirmed by using a non- targeting ‘scramble’ probe. In this case no significant fluorescent signal from the flare strands nor the anchored sense strands were observable (Fig.1).

It is also noteworthy that the localisation of the fluorescent signal from the released flare strands is highly specific and appears to correspond to mitochondrial co-localisation (see section S-VI b and Fig. S7).[21, 22] We regard this finding as vital, as it suggests that some probes were indeed localised within the cytoplasm and not trapped within endosomal compartments of the cells.

It is a well-known phenomenon that most nanoparticles enter cells through endocytosis.[23] Subsequently, particles remain trapped in endosomal compartments without being able to carry out their specifically designed task.

Whilst it has been shown that endosomal escape is possible for certain types of functional nanoparticles, no reports of such a

mechanism for SNA-like nanoparticulate systems have emerged to date.[24] Nevertheless, other groups as well as ourselves have observed that the fluorescence signal obtained from the nanoparticle probes displays mitochondrial co-localisation[22] as well as specificity for the corresponding mRNAs.[25] This observation can only be explained by the presence of some nanoparticles and released flare strands in the cytoplasm. Testing this hypothesis, we analysed several thin sections of cells incubated with the nano-probes by transmission electron microscopy (Fig. S9). The data analysis showed that of ~ 1800 particles counted, ~ 3.6 % were cytosolic after 18 h (see Supplementary info section S-VI c). Despite the low abundance, these particles are thought to be responsible for the highly specific fluorescent signal observed. Nevertheless, it has been reported that endosomes and other intracellular organelles may be major sites of mRNA localisation.[26] Thus, more in-depth studies to elucidate the mechanism for endosomal escape are required and are currently being carried out by our group in order to better understand the fate of most SNAs in cells. This in turn will allow us develop strategies to achieve a higher accumulation of functional particles in the cytosol.

Following successful initial live cell trials, we proceeded to further investigate the drug delivery properties of the nanoparticle systems by incorporating Doxorubicin into Vimentin nano-probes. The successful loading of DOX was assessed by fluorescence melting analysis. Due to the duplex stabilizing properties of DOX, changes in the DNA melting profile can give a good indication of its successful intercalation.[27, 28] Here, a clear shift of ~ + 4 ºC in the melting profile of Vimentin nano-probes (sense-flare duplex) after drug incorporation was observed, implying that DOX had successfully intercalated (Fig. S2). Another important aspect is the retention of the drug within the probe to minimize non-specific release. By monitoring the fluorescence of DOX in the supernatant for up to 48 h, we established that the drug was not released from the probe without specific external stimuli (e.g. heat, denaturation of DNA or displacement of flare strand Fig. S6), thus ensuring minimized collateral damage.

Investigating if these findings also held true in a live cell environment, we analyzed both the model epithelial (16HBE) and mesenchymal (MRC-5) cells for DOX release following incubation with Vimentin nano-probes for 18 h. Having established that the probes for the detection of Vimentin

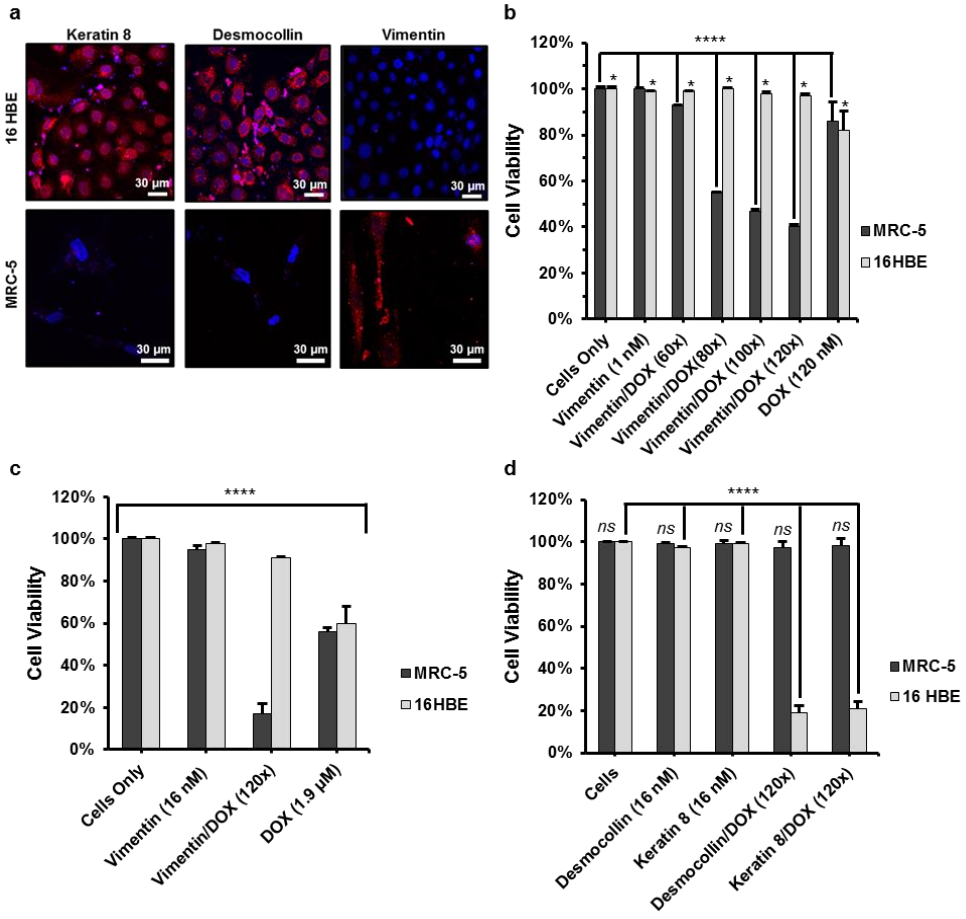


Figure 2. Viability assays of cells incubated with DOX-loaded nano-probes. (a) Confocal micrograph of model epithelial (16 HBE) and mesenchymal (MRC-5) cells after incubation with DOX-loaded nano-probes (for clarity only DOX and nuclear counterstain fluorescence are shown). Colour guide: DOX – red, nuclear counter stain – blue. (b) Viability assays of 16 HBE and MRC-5 cells incubated with Vimentin or Vimentin-DOX nano-probes. The particle concentration was constant at 1 nM, whilst the amount of incorporated DOX was increased. (c) Viability assay of 16 HBE and MRC-5 cells at a particle concentration of 16 nM and maximum DOX loading. (d) Viability assay of 16 HBE and MRC-5 cells incubated with Desmocollin, Keratin 8 or their drug-loaded counterparts. Data are shown as mean ± SEM (*n = 3*). P-values were determined by a one-way ANOVA. *p < 0.05, ****p < 0.0001, *ns* : not significant.

mRNA exhibited activity exclusively in the mesenchymal cells (cf. Fig 1), we concluded that the delivery of DOX using these probes should be equally targeted to the mesenchymal cells. An important element of the success of these experiments is that whilst Doxorubicin intercalates firmly between DNA strands, it cannot intercalate between the base pairs of RNA-DNA hybrid duplexes.[29, 30] This property of Doxorubicin allows the effective release of the drug from the particles in the presence of the specific target mRNA strand. Making use of the inherent fluorescent properties of DOX, we visualized its successful release by confocal microscopy. Figure 2a shows a strong DOX fluorescent signal in the mesenchymal cells (green), whilst no observable signal was detected in the epithelial cells, suggesting that the probes retained their target specificity and released their cargo only in the presence of Vimentin mRNA (i.e. in the mesenchymal cells).

Complementary viability assays further revealed that drug delivery was highly targeted and occurred exclusively in the mesenchymal cell line, achieving an efficient reduction in cell viability. Importantly, we also showed that drug delivery can be regulated by means of an increase in flare strand-sense strand duplexes (Fig. 2b) – thus resulting in higher drug loading – or by simply increasing the nano-probe concentration (Fig. 2c). The highest nano-probe/drug concentration (causing no toxicity to the cells in terms of the nanoparticle content) showed a decrease in cell viability of up to 83%. This is a vast improvement in the efficient delivery of DOX to cells in comparison to its delivery without the use of DNA-nanoparticle probe. To prove that this result was not an artefact of the specific cell line, a control experiment was performed where both Keratin 8 and Desmocollin nano-probes were loaded with DOX in order to deliver the drug solely to the epithelial cell line. Figures 2a and d indicate that, as predicted, the drug was released solely in the epithelial cell line with a maximum cell death potency of up to 85%. These observations suggest that the use of oligonucleotide-gold nanoparticle probes result in a highly increased potency of Doxorubicin compared to the delivery of the free drug, especially if considered that only a very small fraction of particles is active in the cytosol, thus demonstrating the great potential of this method of drug delivery.

Conclusions

In conclusion, we show the first example of this type of functional nanoparticles which can distinguish between different cells and selectively release an anticancer drug only in the model metastatic cells. While there are several examples of nanoparticles used as carriers for drug delivery in cells, this is the first example of nanoparticles that can distinguish between different types of cells based on their mRNA signature and are designed to release their payload only to the cells of interest. The design of intelligent nanoparticulate systems that can selectively perform more than one function is of utmost importance for the development of non-toxic therapies for a broad range of diseases.

Acknowledgements

AGK and TB would like to thank the Leverhulme Trust for funding of the project (ref. RPG-2015-005). ATDBio is gratefully acknowledged for technical support regarding the oligonucleotide synthesis. We thank the staff at the Biomedical Imaging Unit and Primary Ciliary Dyskinesia Research Group, University of Southampton for their assistance and technical support

Notes and references

1 O. Boussif, F. Lezoualch, M. A. Zanta, M. D. Mergny, D. Scherman, B. Demeneix and J. P. Behr, *Proc. Natl. Acad. Sci. U. S. A.*, 1995, **92**, 7297-7301.
2 P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold and M. Danielsen, *Proc. Natl. Acad. Sci. U. S. A.*, 1987, **84**, 7413-7417.
3 N. L. Rosi, D. A. Giljohann, C. S. Thaxton, A. K. R. Lytton-Jean, M. S. Han and C. A. Mirkin, *Science*, 2006, **312**, 1027-1030.
4 L. L. Cummins, S. R. Owens, L. M. Risen, E. A. Lesnik, S. M. Freier, D. McGee, C. J. Guinosso and P. D. Cook, *Nucleic Acids Res.*, 1995, **23**, 2019-2024.
5 D. S. Seferos, D. A. Giljohann, H. D. Hill, A. E. Prigodich and C. A. Mirkin, *J. Am. Chem. Soc.*, 2007, **129**, 15477-15479.
6 J. I. Cutler, E. Auyeung and C. A. Mirkin, *J. Am. Chem. Soc.*, 2012, **134**, 1376-1391.
7 P. C. Patel, D. A. Giljohann, W. L. Daniel, D. Zheng, A. E. Prigodich and C. A. Mirkin, *Bioconjugate Chem.*, 2010, **21**, 2250-2256.
8 C. H. J. Choi, L. L. Hao, S. P. Narayan, E. Auyeung and C. A. Mirkin, *Proc. Natl. Acad. Sci. U. S. A.*, 2013, **110**, 7625-7630.
9 D. Zheng, D. S. Seferos, D. A. Giljohann, P. C. Patel and C. A. Mirkin, *Nano Lett.*, 2009, **9**, 3258-3261.
10 N. Li, H. Yang, W. Pan, W. Diao and B. Tang, *Chem. Commun.*, 2014, **50**, 7473-7476.
11 R. Kalluri, *J. Clin. Invest.*, 2009, **119**, 1417-1419.
12 R. Kalluri and R. A. Weinberg, *J. Clin. Invest.*, 2009, **119**, 1420-1428.
13 M. Zeisberg and E. G. Neilson, *J. Clin. Invest.*, 2009, **119**, 1429-1437.
14 T. Katsumoto, A. Mitsushima and T. Kurimura, *Biology of the Cell*, 1990, **68**, 139-146.
15 J. Ivaska, H.-M. Pallari, J. Nevo and J. E. Eriksson, *Exp. Cell Res.*, 2007, **313**, 2050-2062.
16 A. Satelli and S. Li, *Cell. Mol. Life Sci.*, 2011, **68**, 3033-3046.
17 M. Dauphin, C. Barbe, S. Lemaire, B. Nawrocki-Raby, E. Lagonotte, G. Delepine, P. Birembaut, C. Gilles and M. Polette, *Lung Cancer*, 2013, **81**, 117-122.
18 J. M. Lee, S. Dedhar, R. Kalluri and E. W. Thompson, *J. Cell Biol.*, 2006, **172**, 973-981.
19 C. F. Thorn, C. Oshiro, S. Marsh, T. Hernandez-Boussard, H. McLeod, T. E. Klein and R. B. Altman, *Pharmacogenetics and Genomics*, 2011, **21**, 440-446.
20 D. A. Giljohann, D. S. Seferos, W. L. Daniel, M. D. Massich, P. C. Patel and C. A. Mirkin, *Angew. Chem. Int. Ed. Engl.*, 2010, **49**, 3280-3294.
21 W. J. Rhee and G. Bao, *Nucleic Acids Res.*, 2010, **38**.
22 W. E. Briley, M. H. Bondy, P. S. Randeria, T. J. Dupper and C. A. Mirkin, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 9591-9595.

23 A. E. Nel, L. Madler, D. Velegol, T. Xia, E. M. V. Hoek, P. Somasundaran, F. Klaessig, V. Castranova and M. Thompson, *Nat. Mater.*, 2009, **8**, 543-557.
24 T. F. Martens, K. Remaut, J. Demeester, S. C. De Smedt and K. Braeckmans, *Nano Today*, 2014, **9**, 344-364.
25 A. E. Prigodich, P. S. Randeria, W. E. Briley, N. J. Kim, W. L. Daniel, D. A. Giljohann and C. A. Mirkin, *Anal. Chem.*, 2012, **84**, 2062-2066.
26 M. D. Blower, *Int. Rev. Cel. Mol. Bio.*, 2013, **302**, 1-39.
27 C. M. Alexander, M. M. Maye and J. C. Dabrowiak, *Chem. Commun.*, 2011, **47**, 3418-3420.
28 C. M. Alexander, J. C. Dabrowiak and M. M. Maye, *Bioconjugate Chem.*, 2012, **23**, 2061-2070.
29 J. S. Ren and J. B. Chaires, *Biochemistry*, 1999, **38**, 16067-16075.
30 M. J. Waring, J. B. Chaires and B. A. Armitage, *DNA Binders and Related Subjects*, Springer, 2005.