SUMMARY ~~OF THE INVENTION~~

[0011a] In one aspect, there is provided a composition comprising: (a) a plurality of intact, bacterially derived minicells, each minicell of the plurality encompassing regulatory RNA that is packaged in the minicell, and (b) a pharmaceutically acceptable carrier therefor, wherein (i) the regulatory RNA is selected from the group consisting of antisense ssRNA, ribozyme and decoy RNA, (ii) there is an absence from the minicells of a construct for *in situ* expression of the regulatory RNA, and (iii) the plurality comprises a therapeutically effective amount of the regulatory RNA.

[0012] In accordance with one ~~aspect~~embodiment of the ~~invention~~disclosure, therefore, a composition comprising (a) a plurality of intact minicells, each minicell of the plurality encompassing plasmid-free functional nucleic acid, and (b) a pharmaceutically acceptable carrier therefor. The category of "functional nucleic acid" is illustrated by single-, double-, or multi-stranded DNA or RNA. In one embodiment, minicells of the plurality contain plasmid-free functional nucleic acid that is regulatory RNA. Examples of such regulatory RNA include, but are not limited to,siRNA, miRNA, and shRNA.

[0013] The minicell-packaged functional nucleic acid may target the RNA transcripts encoding a protein that contributes to drug resistance, to apoptosis resistance, or to neoplasticity, *inter alia*. Also, a composition of the ~~invention~~disclosure may further comprise a bispecific ligand comprised, for example, of a first arm specific for a minicell surface structure and a second arm specific for a non-phagocytic mammalian cell surface receptor.

[0013a] In one aspect, there is provided a method of delivering regulatory RNA, comprising the steps of: (a) providing a plurality of intact, bacterially derived minicells in a pharmaceutically acceptable carrier, each minicell of the plurality encompassing regulatory RNA; and (b) bringing minicells of the plurality into contact with mammalian cells such that the mammalian cells engulf minicells of the plurality, whereby the regulatory RNA is released into the cytoplasm of the target cells, wherein (i) the regulatory RNA is selected from the group consisting of antisense ssRNA, ribozyme and decoy RNA, (ii) there is an absence from the minicells of a construct for in situ expression of the regulatory RNA, and (iii) the plurality comprises a therapeutically effective amount of the regulatory RNA.

[0014] In another ~~aspect~~embodiment of the ~~invention~~disclosure, a method is provided for delivering a functional nucleic acid to a target mammalian cell. The inventive methodology comprises (a) providing a plurality of intact minicells in a pharmaceutically acceptable carrier, each minicell of the plurality encompassing plasmid-free functional nucleic acid, and (b) bringing minicells of the plurality into contact with mammalian cells such that the mammalian cells engulf minicells of the plurality, whereby the functional nucleic acid is released into the cytoplasm of the target cells. As mentioned, the functional nucleic acid, exemplified by regulatory RNA such as siRNA, miRNA and shRNA, can target RNA transcripts encoding a protein that contributes to drug resistance, apoptosis resistance or neoplasticity. In other embodiments, the methodology of the ~~invention~~disclosure further comprises delivering a drug, distinct from the functional nucleic acid, to the target mammalian cell. The drug can be administered after or concurrently or even before the administration of the minicell composition.

[0014a] In one aspect, there is provided a method for formulating the composition as defined herein, comprising co-incubating a plurality of intact, bacterially derived minicells and the regulatory RNA in a buffer.

[0015] In accordance with another ~~aspect~~embodiment, the present ~~invention~~disclosure contemplates a method for formulating a minicell with a plasmid-free functional nucleic acid. The method comprises co-incubating a plurality of minicells with a functional nucleic acid, such as regulatory RNA like siRNA, miRNA or shRNA, in a buffer. In some embodiments, the co-incubation may involve gentle shaking, while in others the co-incubation is static. In some ~~aspects~~embodiments, the co-

incubation lasts about half an hour, while in others it lasts about an hour. In one embodiment, the buffer comprises buffered saline, for example, a 1X phosphate buffer solution. In another embodiment, the co-incubation is conducted at a temperature of about 4°C to about 37°C, about 20°C to about 30°C, about 25°C, or about 37°C. The co-incubation can comprise about 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12} or 10^{13} minicells.

[0016] Other objects, features and advantages will become apparent from the following detailed description. The detailed description and specific examples are given for illustration only since various changes and modifications within the spirit and scope of the ~~invention~~disclosure will become apparent to those skilled in the art from this detailed description. Further, the examples demonstrate the principle of the ~~invention~~disclosure and cannot be expected to specifically illustrate the application of this ~~invention~~disclosure to all the examples where it will be obviously useful to those skilled in the prior art.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] Figure 1 depicts intact minicells packaged with Cy3 fluorophore-labeled siRNA. Figure 1A is from a light microscope, while Figure 1B shows the same slide but viewed under fluorescent light with a 515-560 excitation filter revealing strongly fluorescent siRNA molecules coincident with the minicells.

[0018] Figure 2 is an image captured by fluorescence confocal microscopy and shows the adhesion and internalization of EGFR-targeted, siRNA-Plk1-packaged minicells into human breast cancer cells *in-vitro*.

[0019] Figure 3 graphically shows a significant anti-tumor effect achieved by treating human breast cancer (MDA-MB-468) xenografts in nude mice with EGFR-targeted, KSP-siRNA-packaged minicells. Control group 1(—◆—) received sterile saline, while experimental group 2 (—⊖—) received $^{EGFR}minicells_{siRNA\text{-}KSP}.(10^9)$, four times per week.

[0020] Figure 4 graphically depicts a significant anti-tumor effect achieved by treating human colon cancer (HCT116) xenografts in nude mice with EGFR-targeted, KSP-siRNA-packaged minicells in conjunction with EGFR-targeted, carboplatin-packaged minicells. Group 1 (—◆—) mice received sterile saline, and mice of groups 2 (- -△- -), 3 (—■—) and 4

incubation was carried out for 2 hours at 37°C, with gentle mixing. Control minicells were mock loaded by incubation with 1 x PBS alone. Post loading minicells were pelleted and washed twice with 1 x PBS by centrifugation for 10 minutes at 16,200 x *g*. Experimental and control minicells were observed under a fluorescence DMLB microscope, product of Leica (Germany) with attached D70 camera, product of Olympus Microscopes (Germany). Images were acquired using 100x oil immersion lens.

[0155] The above co-incubation experiments were also carried out under different experimental conditions such as incubation at room temperature, 37°C, and 4°C. Additionally, the co-incubation times were varied to include 1 hour, 2 hours, 4 hours, and 12 hours, respectively.

[0156] As shown in Figure 1B, the intact the siRNA molecules rapidly diffused into the minicells. The 2-hour incubation at 37°C was sufficient to achieve highly significant packaging of the minicells.

[0157] In order to determine whether the siRNA molecules were inside the minicells or adherent on minicell surfaces, the minicells with Cy3-fluorescence-labeled siRNA were incubated with exonucleases overnight, followed by a repeat of fluorescence microscopy. The results were identical to those shown in Fig. 1B, indicating that the siRNAs were internalized by the minicells and were not adherent on the minicell surface.

2. *In vitro* transfection of human breast cancer cells with regulatory RNA-packaged, bispecific antibody-targeted minicells

[0158] To demonstrate that minicells carrying regulatory RNA are stable in serum *in-vitro* and that they can be internalized by specifically-targeted mammalian cells, the following experiment was carried out.

[0159] siRNA directed to polo-kinase 1 (Plk1) was synthesized with a target sequence 5'-GGTGGATGTGTGGTCCATTTT-3'(SEQ ID NO: 1), and tagged with a fluorescent tag, AlexaFluor 488. Polio kinases have multiple functions during the entry into mitosis, centrosome maturation, bipolar spindle formation, the segregation of chromosomes and cytokinesis, and, crucially, the fidelity monitoring of checkpoint control (Glover *et al.*, 1998; Barr *et al.*, 2004; van de Weerdt and Medema 2006). In humans, Plk1 is the best characterized member of this family.

sister chromatids during mitosis (Enos and Morris, 1990; Blangy et al., 1995; Sawin and Mitchison, 1995; Dagenbach and Endow, 2004). Inhibition of KSP causes the formation of monopolar mitotic spindles, activates the spindle assembly checkpoint, and arrests cells at mitosis, leading to subsequent cell death (Blangy et al., 1995, Caner et al., 1999; Kapoor et al., 2000; Tao et al., 2005).

[0164] An siRNA against KSP was selected for packaging in minicells, to inform the optimization of siRNA extraction from minicells in accordance with the invention. More specifically, KSP-1-siRNA double-stranded oligonucleotide sequences (sense strand; 5'-AAC TGG ATC GTA AGA AGG CAG-3' SEQ ID NO: 2) were synthesized and packaged into minicells, pursuant to the procedures set out in Example 1, *supra*.

[0165] $Minicells_{siRNA\text{-}KSP}$ (10^{10}) and a comparable number of control empty minicells were processed, using a number of commercially available nucleic acid extraction kits. The results showed that the mirVana miRNA isolation kit (Ambion) provided quantitative extraction of the siRNA-KSP from intact minicells. The procedure was carried out according to the manufacturer's instructions.

[0166] The purified siRNA first was stained with an ultra-sensitive fluorescent nucleic acid dye, RiboGreen™, product of Molecular Probes Inc. (Eugene, Oregon USA), followed by quantitation using the NanoDrop ND-3300 Fluorospectrometer, product of NanoDrop Technologies Inc. (Wilmington, Delaware USA), also according to the manufacturer's instructions. RNA-bound RiboGreen™ has an excitation maximum of ~500nm and an emission maximum of ~525nm.

[0167] The results showed that the minicells were able to carry the siRNAs. 10^{10} empty minicells carried ~1.4 μg RNA, presumably a background level of endogenously formed bacterial RNA. The same number of $minicells_{siRNA\text{-}KSP}$ carried ~2.7 μg RNA, comprising endogenous bacterial RNA plus exogenously packaged siRNA-KSP Thus, these data demonstrate that 10^{10} minicells can package at least ~1.3 μg of exogenously packaged siRNA.

4. *In vivo* demonstration of anti-tumor effects achieved by regulatory RNA-packaged minicells

stable within the minicells *in vivo*, (b) intact and fully functional siRNAs were delivered to the tumor cells *in vivo*, and (c) the minicell delivered therapeutically significant concentrations of siRNA to the tumor cells *in vivo*.

5. Demonstration of tailor-made cancer therapy via treatment with regulatory RNA-packaged minicells, followed by drug-packaged minicells

[0174] Most anti-cancer therapies are associated with drug resistance. The same is true for regulatory RNA treatments, since genetic mutations in tumor cells can render the regulatory RNA ineffective if the target gene mutates within the sequence targeted by the regulatory RNA.

[0175] There has been no effective strategy to address drug resistance in cancer patients. Instead, new drugs must be administered to bypass the mutation. This approach faces serious difficulties, however, since most anti-cancer drugs are highly toxic, and combined therapies augment that toxicity, resulting in dose limitation and frequent abandonment of therapy when the patient can no longer cope with the toxicity. The following study was conducted to assess the efficacy of regulatory RNA-packaged minicells in addressing such resistance.

[0176] As described above, minicells were purified and packaged (10^9) with anti-KSP or anti-Plk1 siRNA. Also as previously described, bispecific antibody was prepared, carrying anti-*S. typhimurium* O-antigen and anti-human EGFR specificities, and was attached to the $minicells_{siRNA\text{-}KSP}$ to generate $^{EGFR}minicells_{siRNA\text{-}KSP}$.

[0177] Human colon cancer (HCT116; ATCC) xenografts were established in nude mice, as described in Example 3, and were treated i.v. as follows: Group 1 mice received sterile saline, and mice of groups 2, 3 and 4 were treated for the first 10 doses (see Figure 4) with 10^9 $^{EGFR}minicells_{siRNA\text{-}Plk1}$, $^{EGFR}minicells_{siRNA\text{-}KSP\text{-}1}$, and $^{EGFR}minicells_{siRNA\text{-}KSP\text{-}2}$, respectively. The Plk1 and KSP-1 sequences were as shown in the examples above. siRNA-KSP-2 (sense strand; 5' CTGAAGACC TGAAGACAAT 3' SEQ ID NO: 3) targets a different segment of KSP mRNA. After day 33, mice in groups 2, 3 and 4 were treated with two doses of $^{EGFR}minicells_{carboplatin}$.

[0178] The results showed (Figure 4) that post-day 26, the tumors were becoming resistant to the siRNA treatments. Accordingly, the mice in groups 2, 3 and 4 were treated for the four

~~WE CLAIM:~~

1. A composition comprising (a) a plurality of intact minicells, each minicell of said plurality encompassing plasmid-free functional nucleic acid, and (b) a pharmaceutically acceptable carrier therefor.

2. The composition of claim 1, wherein said plurality contains a therapeutically effective amount of said functional nucleic acid.

3. The composition of claim 1, wherein said functional nucleic acid is a regulatory RNA.

4. The composition of claim 3, wherein said regulatory RNA is selected from the group consisting of siRNA, miRNA and shRNA.

5. The composition of claim 1, wherein said functional nucleic acid is not chemically modified.

6. The composition of claim 1, wherein said functional nucleic acid is DNA.

7. The composition of claim 1, wherein said functional nucleic acid targets the RNA transcript encoding a protein that contributes to drug resistance.

8. The composition of claim 1, wherein said functional nucleic acid targets the transcript of P-glycoprotein, MDR-2, or MDR-3.

9. The composition of claim 1, wherein said functional nucleic acid targets the transcript of MRP2, BCR-ABL, STI-571 resistance-associated protein, lung resistance-related protein, cyclooxygenase-2, nuclear factor kappa, XRCC1, ERCC1, GSTP1, mutant β-tubulin, or a growth factor.

10. The composition of claim 1, wherein said functional nucleic acid targets the RNA transcript encoding a protein that contributes to apoptosis resistance.

11. The composition of claim 10, wherein said functional nucleic acid targets a transcript of Bcl-2, Bcl-X_L, A1/Bfl 1, focal adhesion kinase or p53 protein.

12. The composition of claim 1, wherein said functional nucleic acid targets a RNA transcript of encoding a protein that contributes to neoplasticity.

13. The composition of claim 12, wherein said functional nucleic acid targets a transcript of β-Catenin, PKC-α, C-RAF, K-Ras, DP97 Dead box RNA helicase, cdk1, DNMT1, FLIP, C-Sfc, 53BPI, Polycomb group protein EZH2, ErbB1, HPV-16 E5 and E7, Fortilin & MCI1P, KSP, DIP13α, MBD2, p21, KLF4, tpt/TCTP, SPK1 & SPK2, P300, PLK1, Trp53, Ras, ErbB1, VEGF, or BAG-1.

14. The composition of claim 1, further comprising a drug.

15. The composition of claim 14, wherein said functional nucleic acid targets the transcript encoding a protein that contributes to resistance to said drug.

16. The composition of claim 14, wherein said drug is packaged in an intact minicell.

17. The composition of claim 16, wherein said functional nucleic acid and said drug are packaged within the same minicell.

18. The composition of claim 1, further comprising a bispecific ligand.

19. The composition of claim 18, wherein said bispecific ligand comprises a first arm that carries specificity for a minicell surface structure and a second arm that carries specificity for a non-phagocytic mammalian cell surface receptor.

20. The composition of claim 19, wherein said minicell surface structure is an O-polysaccharide component of a lipopolysaccharide on said minicell surface.

21. The composition of claim 19, wherein said mammalian cell surface receptor is capable of activating receptor-mediated endocytosis of said minicell.

22. The composition of claim 18, wherein said bispecific ligand comprises an antibody or antibody fragment.

23. The composition of claim 1, wherein said composition contains fewer than about 1 contaminating parent bacterial cell per 10^7 minicells.

24. The composition of claim 1, wherein said composition contains fewer than about 1 contaminating parent bacterial cell per 10^8 minicells

25. The composition of claim 1, wherein said composition contains fewer than about 1 contaminating parent bacterial cell per 10^9 minicells

26. The composition of claim 1, wherein said composition contains fewer than about 1 contaminating parent bacterial cell per 10^{10} minicells

27. The composition of claim 1, wherein said composition contains fewer than about 1 contaminating parent bacterial cell per 10^{11} minicells.

28. A method of delivering functional nucleic acid, comprising (a) providing a plurality of intact minicells in a pharmaceutically acceptable carrier, each minicell of said plurality encompassing plasmid-free functional nucleic acid, and (b) bringing minicells of said plurality into contact with mammalian cells such that said mammalian cells engulf minicells of said plurality, whereby said functional nucleic acid is released into the cytoplasm of the target cells.

29. The method of claim 28, wherein said functional nucleic acid is a regulatory RNA.

30. The method of claim 29, wherein said regulatory RNA is selected from the group consisting of siRNA, miRNA and shRNA.

31. The method of claim 28, wherein said functional nucleic acid is DNA.

32. The method of claim 28, wherein contact between said minicells and said mammalian cells occurs *in vitro*.

33. The method of claim 28, wherein contact between said minicells and said mammalian cells occurs *in vivo*.

34. The method of claim 33, wherein at least a portion of said plurality comprises a therapeutically effective amount of said functional nucleic acid.

~~35. The method of claim 28, wherein said functional nucleic acid targets the transcript~~ encoding a protein that contributes to drug-resistance.

36. The method of claim 28, wherein said functional nucleic acid targets the transcript of P-glycoprotein, MDR-2 or MDR-3.

37. The method of claim 28, wherein said functional nucleic acid targets the transcript of MRP2, BCR-ABL, STI-571 resistance-associated protein, lung resistance-related protein, cyclooxygenase-2, nuclear factor kappa, XRCC1, ERCC1, GSTP1, mutant β-tubulin, or a growth factor.

38. The method of claim 28, wherein said functional nucleic acid targets the transcript of a protein that contributes to apoptosis resistance.

39. The method of claim 38, wherein said functional nucleic acid targets a transcript of Bcl-2, Bcl-X_L, A1/Bfl 1, focal adhesion kinase or p53 protein.

40. The method of claim 28, wherein said functional nucleic acid targets a transcript of a protein that contributes to neoplasticity.

41. The method of claim 40, wherein said functional nucleic acid targets a transcript of β-Catenin, PKC-α, C-RAF, K-Ras, DP97 Dead box RNA helicase, cdk1, DNMT1, FLIP, C-Sfc, 53BPI, Polycomb group protein EZH2, ErbB1, HPV-16 E5 and E7, Fortilin & MCI1P, DIP13α, KSP, MBD2, p21, KLF4, tpt/TCTP, SPK1 & SPK2, P300, PLK1, Trp53, Ras, ErbB1, VEGF, or BAG-1.

42. The method of claim 28, further comprising a step (c) of delivering a drug to said target mammalian cell.

43. The method of claim 42, wherein said functional nucleic acid targets the transcript of a protein that contributes to resistance to said drug.

44. The method of claim 42, wherein said drug is packaged in an intact minicell.

45. The method of claim 44, wherein said functional nucleic acid and said drug are packaged within the same minicell.

46. The method of claim 42, wherein step (c) occurs subsequent to steps (a) and (b).

47. The method of claim 42, wherein said drug is delivered concurrently with step (b).

48. The method of claim 28, wherein step (b) entails bringing a bispecific ligand into contact with at least some of said intact minicells and said target mammalian cell, such that said bispecific ligand causes minicells to bind to said mammalian cell.

49. The method of claim 48, wherein said target mammalian cell is a non-phagocytic cell.

50. The method of claim 48, wherein said bispecific ligand comprises a first arm that carries specificity for a minicell surface structure and a second arm that carries specificity for a non-phagocytic mammalian cell surface receptor.

51. The method of claim 50, wherein said minicell surface structure is an O-polysaccharide component of a lipopolysaccharide on said minicell surface.

52. The method of claim 50, wherein said mammalian cell surface receptor is capable of activating receptor-mediated endocytosis of said minicell.

53. The method of claim 48, wherein said bispecific ligand comprises an antibody or antibody fragment.

54. The method of claim 28, wherein said mammalian cell is phagocytosis- or endocytosis-competent.

55. A method for formulating a minicell of the composition of claim 1, comprising co-incubating a plurality of intact minicells with functional nucleic acid in a buffer.

56. The method of claim 55, wherein said functional nucleic acid is a regulatory RNA.

57. The method of claim 55, wherein said regulatory RNA is selected from the group consisting of siRNA, miRNA and shRNA.

58. The method of claim 55, wherein said functional nucleic acid is DNA.

59. The method of claim 55, wherein said co-incubation comprises gentle shaking.

60. The method of claim 55, wherein said co-incubation lasts about 0.5 hour.

61. The method of claim 55, wherein said co-incubation lasts about one hour.

62. The method of claim 55, wherein said buffer comprises buffered saline.

63. The method of claim 62, wherein said buffered saline is in gelatin form.

64. The method of claim 55, wherein said buffer comprises a 1X phosphate buffer solution.

65. The method of claim 55, wherein said co-incubation is conducted at a temperature of about 4°C to about 37°C.

66. The method of claim 55, wherein said co-incubation is conducted at a temperature of about 20°C to about 30°C.

67. The method of claim 55, wherein said co-incubation is conducted at a temperature of about 25°C.

68. The method of claim 55, wherein said co-incubation is conducted at a temperature of about 37°C.

69. The method of claim 68, wherein said co-incubation comprises about 10^{10} minicells.

70. The method of claim 55, wherein said co-incubation comprises about 10^{7}, 10^{8}, 10^{9}, 10^{10}, 10^{11},10^{12} or 10^{13} minicells.

71. The method of claim 55, wherein said co-incubation comprises about 10^{10} minicells.

WE CLAIM:

1. A composition comprising:

(a) a plurality of intact, bacterially derived minicells, each minicell of the plurality encompassing regulatory RNA that is packaged in the minicell, and

(b) a pharmaceutically acceptable carrier therefor,

wherein (i) the regulatory RNA is selected from the group consisting of antisense ssRNA, ribozyme and decoy RNA, (ii) there is an absence from the minicells of a construct for *in situ* expression of the regulatory RNA, and (iii) the plurality comprises a therapeutically effective amount of the regulatory RNA.

2. The composition of claim 1, wherein the regulatory RNA is not chemically modified.

3. The composition of claim 1, wherein the regulatory RNA specifically interferes with expression of a protein that contributes to drug resistance.

4. The composition of claim 3, wherein the protein is P-glycoprotein, MDR-2, or MDR-3.

5. The composition of claim 3, wherein the protein is MRP2, BCR-ABL, STI-571 resistance-associated protein, lung resistance-related protein, cyclooxygenase-2, nuclear factor kappa, XRCC1, ERCC1, GSTP1, mutant β-tubulin, or a growth factor.

6. The composition of claim 1, further comprising a bispecific ligand.

7. The composition of claim 6, wherein the bispecific ligand comprises a first arm that carries specificity for a minicell surface structure and a second arm that carries specificity for a non-phagocytic mammalian cell surface receptor.

8. The composition of claim 7, wherein the mammalian cell surface receptor is capable of activating receptor-mediated endocytosis of the minicell.

9. The composition of claim 1, wherein the composition comprises fewer than about 1 contaminating parent bacterial cell per 10^{10} minicells.

10. The composition of claim 1, wherein the composition comprises fewer than about 1 contaminating parent bacterial cell per 10^{11} minicells.

11. The composition of claim 1, wherein the regulatory RNA is antisense ssRNA.

12. A method of delivering regulatory RNA, comprising the steps of:

(a) providing a plurality of intact, bacterially derived minicells in a pharmaceutically acceptable carrier, each minicell of the plurality encompassing regulatory RNA; and

(b) bringing minicells of the plurality into contact with mammalian cells such that the mammalian cells engulf minicells of the plurality, whereby the regulatory RNA is released into the cytoplasm of the target cells,

wherein (i) the regulatory RNA is selected from the group consisting of antisense ssRNA, ribozyme and decoy RNA, (ii) there is an absence from the minicells of a construct for *in situ* expression of the regulatory RNA, and (iii) the plurality comprises a therapeutically effective amount of the regulatory RNA.

13. The method of claim 12, wherein contact between the minicells and the mammalian cells occurs *in vitro*.

14. The method of claim 12, wherein contact between said minicells and said mammalian cells occurs *in vivo*.

15. A method for formulating the composition of claim 1, comprising co-incubating a plurality of intact, bacterially derived minicells and the regulatory RNA in a buffer.

16. The method of claim 15, wherein said regulatory RNA is antisense ssRNA.