1. Introduction

Many drugs act on intracellular targets and require efficient endocytosis and permeation to the site of action in specific organelle in order to exert their pharmacological effects. Complexity of the cellular endocytosis and trafficking pathways [1] and high compartmentalization of the cells into different organelles leads to suboptimal magnitude and duration of pharmacological effects at the organelle of interest as well as to non-specific effects due to exposure of additional organelles to the drug. Therefore, encapsulation of the intracellularly-acting drugs into specialized drug delivery systems (DDSs) that are targeted to specific organelle and deliver the drug in a controlled fashion is required in order to attain efficient and selective pharmacological effects [2,3]. Intracellularly-targeted DDSs can be based on drug-encapsulating particles or vesicles (liposomes) decorated with organelle-specific targeting moieties. Efficient targeting of the drug to the organelle of interest requires recognition of the targeting moieties by the endogenous intracellular trafficking mechanisms [2,3].

Feasibility of targeted delivery of drugs and model compounds into individual organelles has been assessed in several studies [reviewed in [4]] that claimed preferential drug delivery to target organelles. Targeting moieties that were used for this purpose included: (1) peptide sequences that are recognized by the cytosolic transport systems of the host cell, such as endoplasmic reticulum (ER) signal peptide or ER-retrieval sequence, nuclear localization signal, mitochondrial localization signal, etc.; (2) peptide or non-peptide molecules that preferentially interact with the membrane of the target organelle, e.g., mitochondrialotropic arginine-rich peptides or positively charged compounds. However, the...
above-mentioned studies provide limited quantitative and mechanistic insights into effect of organelle-specific targeting residues on the intracellular trafficking of nanoparticle formulations and its effect on the resulting pharmacological activities of the drug. Efficient intracellular targeting of a drug should lead to preferential accumulation of the drug in the target organelle, and not in the other intracellular organelles, following endocytosis. Assessment of drug amounts that have reached the individual organelles, i.e., the drug intracellular distribution, can be done using fluorescence-based (confocal microscopy) [5] and biochemical [6,7] approaches. However, both these approaches are limited to semi-quantitative analysis of drug content in selected set of organelles, and do not allow detailed analysis of the drugs intracellular distribution and mass balance calculations. Therefore, studies on intracellular drug targeting usually report relative drug accumulation in the target organelle, but not in other organelles. In some studies, intracellular drug concentrations are not quantified, and the extent of drug accumulation in the target organelle is deduced from the bioactivity of the organelle-targeted vs. untargeted formulations. In both cases, efficiency of drug targeting to a specific organelle (i.e., drug/DDS accumulation in the target organelle in comparison to the other organelles) cannot be readily estimated.

In this research project we sought to develop tools for quantitative assessment of intracellular localization of nanoparticulate DDS and use them to assess the efficiency of intracellular targeting based on decoration of these DDS with targeting residues. To this end, we developed novel 'IntraCell' plugin for ImageJ for quantitative analysis of confocal images and applied it to assess the in vitro intracellular targeting efficiency of novel DDS (see Fig. 1). The developed plugin is based on threshold-based identification of borders of cell and of the individual organelles on confocal images and pixel-by-pixel analysis of fluorescence intensities. Previously, we successfully applied similar pixel-by-pixel analysis protocol to quantify fluorescence resonance energy transfer (FRET) efficiency in non-continuous organelles, such as ER [8].

We choose to assess DDS targeting to the ER since this organelle is a major site of peptide loading on the MHC class I molecules [9], and efficient delivery of exogenous peptide (e.g., immunogenic SIINFEKL peptide) to this organelle can dramatically enhance its cross-presentation efficiency for the purpose of anti-cancer vaccination [10,11]. As a targeting signal we used peptides containing specific ER-targeting moieties (KXXX signal) that were previously shown to target intracellular proteins to the ER [12,13].

2. Materials and methods

2.1. Materials, antibodies and cells

Poly(DL lactide-co-glycolide) polymer (PLGA, 50:50 monomers ratio, with free carboxylic end groups, MW 31–58 kDa) was from LACTEL (DURECT Corp., USA). SIINFEKL, branching (ADGADGADG), and propionic-acid N-conjugated ER-targeting (AAKKA) and control (KAAAAK) peptides were synthesized by GL Biochem, China. Bovine serum albumin labeled with fluorescein isothiocyanate (BSA-FITC), poly(ethylene-alt-maleic anhydride) (PEMA), N-(3-Dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (EDC), N-hydroxy-succinimide (NHS), and 2-(N-morpholino)ethanesulfonic acid (MES) were from Sigma–Aldrich (Rehovot, Israel). DMSO, ethanol, methanol, isopropanol, dichloromethane, and other analytical grade solvents were from BioLab, Israel. All other reagents were of analytical grade.

The following antibodies were used: mouse monoclonal antibody against EEA1 (7051, Abcam), Rabbit anti-calreticulin polyclonal antibody (PA3-900, Thermo Scientific), Alexa Fluor 546 F(ab) 2 fragment of goat anti-mouse IgG (Invitrogen), Alexa Fluor 633 goat anti-rabbit IgG (Invitrogen).

HeLa human cervical carcinoma cells were cultured in DMEM medium (Biological Industries, Beit-Haemek, Israel) supplemented with 5% fetal bovine serum, 2 mM l-glutamine, 1 mM pyruvate, 1% non-essential amino acids, 100 U/ml penicillin and 0.1 mg/ml streptomycin. The cells were grown in a humidified atmosphere with 5% CO2 and 37 °C, and undergo incubation with the studied formulations at the same conditions (see below).

2.2. Nanoparticle preparation

Nanoparticles were prepared by a double emulsion technique. Solution of BSA–FITC and SIINFEKL in PBS (2 mg/ml of each component, 200 µl) or PBS (to generate empty nanoparticles, a negative control) was added to solution of PLGA in dichloromethane (100 mg/ml, 2 ml) and sonication was performed using Vibracell probe sonicator (Sonics, CT, USA) with probe No. 3 for 2 min on ice. To the resulting o/w emulsion aqueous solution of PEMA (20 mg/ml, saturated with DCM, 5 ml) was added and w/o/w emulsion was generated by sonication for 5 min at the same conditions. The generated emulsion was transferred to aqueous solution of PEMA with isopropanol (2.7 mg/ml and 100 mg/ml, respectively) and stirred until evaporation of organic solvents. The nanoparticles were sedimented by centrifugation, washed, resuspended in DDW and lyophilized using FreeZone 2.5 Plus Lyophilizer (Labconco, MO, USA).

2.3. Decoration of the nanoparticles with targeting residues

At the first stage, branching peptide was conjugated to the nanoparticles’ surface using carbodiimide reaction. The nanoparticles were resuspended in MES buffer (100 mM, pH 5.8) and undergo reaction with EDC and NHS (10 M and 5 M, respectively) for 30 min at room temperature. The nanoparticles with activated carboxylic groups were washed, resuspended in borate buffer (200 mM, pH 8.5), undergo reaction with branching peptide for 2 h at room temperature.

At the next stage, linker (3-azidopropylamine) was conjugated using carbodiimide reaction at the same conditions. The linker was synthesized from sodium azide and 3-chloropropylamine according to the procedure described by Jiang et al. [14].

At the last stage, ER-targeting or control peptide were conjugated to the linker using Click reaction. The nanoparticles decorated with the branching peptide and linker were resuspended in aqueous solution of copper sulfate and sodium ascorbate (100 mM and 500 mM, respectively), propionic-acid N-conjugated ER-targeting or control (scrambled) peptide were added, and the suspension was incubated for 3 h at room temperature with constant stirring. The nanoparticles were sedimented by centrifugation, washed, resuspended in DDW and lyophilized.
2.4. Characterization of nanoparticles

Nanoparticle morphology was studied by scanning electron microscopy (SEM). Sample of the lyophilized formulations were placed on carbon adhesive tape, coated with gold, and imaged using Quanta 200 scanning electron microscope (Hillsbro, OR, USA) at the Institute of Applied Research (Ben-Gurion University, Beer-Sheva, Israel). Quantitative analysis of SEM images was performed using ImageJ software (version 1.40C, NIH, USA [15]). Nanoparticle size and ζ-potential were measured using ZetaPlus instrument (Brookhaven Instruments Corporation Ltd., NY, USA) at the National Institute of Biotechnology (Ben-Gurion University, Beer-Sheva, Israel).

The encapsulation efficiency was determined by analysis of supernatants of nanoparticle suspensions using QuantPro BCA assay kit (Sigma). In vitro release rate from the nanoparticles into PBS solution (1 mM, pH 7.4) at 37 °C was determined using the same kit. Supernatants collected from empty nanoparticles served as control.

2.5. Uptake of the nanoparticles by HeLa cells in vitro and analysis of intracellular localization

HeLa cells grown on glass coverslips were incubated with the individual formulations for 6 h, extensively washed with PBS, fixed with 2.5% formaldehyde in PBS, permeabilized with 0.1% Triton X-100 solution, stained simultaneously with antibodies against EEA1 and calreticulin followed by secondary antibodies, and mounted on slides using Mowiol 4-88 with DABCO anti-fading agent. Cells stained with EEA1 or calreticulin antibodies separately, secondary antibodies only, and/or incubated with empty nanoparticles were used as controls.

Representative images of the prepared slides at the individual fluorescence channels were sequentially collected with Olympus FV100-DX81 confocal microscope (Tokyo, Japan) equipped with 60× oil objective. All experimental samples were imaged on the same day using a constant set of imaging parameters (that were initially adjusted to keep all the samples' fluorescence within a linear range). The collected images were analyzed using a custom-written ‘IntraCell’ plugin (freely available at ImageJ software repository [16]), to identify the borders of the individual cells and the organelles (early endosomes and endoplasmic reticulum), and to quantify the relative NP fluorescence inside and outside these organelles within a specific cell. For each formulation, 25–30 cells were analyzed using this approach.

2.6. Statistical analysis

The data are presented as mean ± standard deviation. Differences in the studied parameters between the experimental groups were analyzed using ANOVA with Tukey–Kramer post-test using InStat 3.0 software (GraphPad Software Inc.). The p value less than 0.05 was termed significant.

3. Results

3.1. Nanoparticle preparation and characterization

Spherical nanoparticles with narrow size distribution were prepared (Figs. 2A and B). The diameter of unconjugated nanoparticles was 320 ± 92 nm and the ζ-potential was −32 ± 1 mV, which is consistent with presence of free carboxylic groups on the nanoparticles' surface. The generated nanoparticles efficiently encapsulated the antigenic peptide and marker molecules: the encapsulation efficiency of unconjugated nanoparticles was 33 ± 8% (mean ± SD). The in vitro release of the encapsulated material exhibited biphasic pattern: ‘burst effect’ (~30% of the content) during the first hours was followed by gradual release over 2–3 days (Fig. 2C). Step-wise decoration of the generated nanoparticles with branching peptide, linker, and the targeting or control peptides was associated with characteristic changes of the FTIR spectra and the ζ-potential of the nanoparticles, but did not affect their size and morphology (data not shown).

3.2. Nanoparticle uptake and intracellular trafficking

HeLa cells efficiently endocytosed nanoparticles (see Fig. 3). The intracellular NP fluorescence was affected by the conjugation procedure, and NP fluorescence in cells incubated with unconjugated NPs was approximately 2-fold higher as compared to the cells incubated with the conjugated NPs (see Fig. 4A). This outcome apparently does not indicate differences in the uptake efficiencies of the studied formulations, but reflects lower content of the fluorescent probe in the control and targeting peptide-conjugated NPs due to its partial release (the burst effect) during the multi-stage conjugation procedure.

Quantitative analysis of the images was performed using ‘IntraCell’ ImageJ plugin. The threshold values of fluorescence (for detection of borders of the cells, the ER and endosomal compartments, see Fig. 3C) were set based on the background fluorescence in the control samples and were kept constant for all the analyzed images. Image analysis with ‘IntraCell’ plugin revealed high colocalization of the nanoparticles with the ER and endosomes and significant differences in the intracellular trafficking patterns of the studied nanoparticle formulations. Targeting peptide-conjugated nanoparticles showed significantly higher accumulation in the ER (0.47 ± 0.34) and endosomes (0.26 ± 0.24), as compared to the unconjugated nanoparticles (0.20 ± 0.15 and 0.10 ± 0.06, respectively, see Fig. 4B). Control peptide-conjugated nanoparticles accumulated in the ER and endosomes (0.40 ± 0.28 and 0.22 ± 0.17, respectively) was intermediate, and no statistically-significant differences to the other formulations was observed due to high variability in the intracellular localization of nanoparticles in the individual cells.

4. Discussion

4.1. Advantages and limitations of the developed plugin

Efficient intracellular targeting of drugs and DDS is a major challenge that should be overcome to enhance the therapeutic efficiency of biopharmaceuticals and other intracellularly-acting drugs. Studies that quantitatively assess the mechanisms, barriers, and efficiency of intracellular drug delivery are required to determine the therapeutic potential of intracellular targeting of nano-delivery systems. In this study we report development and application of a novel plugin that is useful for quantitative analysis of intracellular localization of the drug/DDS and estimation of targeting efficiency. The developed ‘IntraCell’ plugin is convenient in use and allows rapid analysis of experimental data from single images or stacks of images (slices). The analysis requires set of images taken from the same field of view with individual images representing fluorescence of the studied formulation and of the individual organelles. In addition, cell borders should be identified using membrane-specific dyes, based on the cells' autofluorescence, or from differential interference contrast (DIC) image. For collection of the required images, the drug or DDS can be labeled with fluorescent dye, and the studied organelles can be fluorescently-labeled using immuno-staining with specific antibodies or organelle-specific dyes. In our studies, we used three fluorophores: BSA-FITC...
encapsulated into the DDS and simultaneous immuno-staining of early endosomes and ER organelles. Generally, up to five labels per sample can be simultaneously used in the modern confocal microscopes due to spectral overlap between the fluorophores. Therefore, the applied analysis approach can be potentially expanded for quantitative assessment of the fluorescently-labeled drug or DDS in up to four individual organelles (current version of ‘IntraCell’ plugin is limited to two organelles).

The applied analysis algorithm is based on pixel selection and pixel-to-pixel analysis of the experimental images. User-defined thresholds for pixel selection are applied in ‘IntraCell’ plugin to identify the borders of the studied organelles and to exclude background pixels from the analysis. The same approach was successfully applied by us previously to separate the background pixels from analysis of FRET efficiency in non-continuous intracellular compartments, such as ER organelle [8], and by other researchers.

Fig. 2. Characterization of the generated nanoparticle formulation (unconjugated nanoparticles): morphology (A), particle size distribution (B), and in vitro release of the encapsulated materials (C). Representative data from three experimental sets for each analysis. The error bars represent SD of triplicates.

Fig. 3. Confocal analysis of the nanoparticle intracellular localization in HeLa cells. Representative images from cells incubated with targeting peptide-conjugated nanoparticles (NPs). The cells were incubated for 6 h with the studied formulations, were stained with antibodies against proteins residing in the early endosomes (ES) or ER, and were imaged using confocal microscope (A). Image overlays (B), identification of the cell borders and organelles (C), yellow and red pixels, respectively) and image analysis were performed using ImageJ software and ‘IntraCell’ plugin.
for studying fluorescence colocalization. Use of thresholds and pixel-based analysis allows convenient and rapid analysis of DDS accumulation in the studied organelles on the acquired images. However, the precision and robustness of this algorithm is limited by image resolution that stems from the technical limitations of a specific confocal microscope, cell dimensions and organelles' shapes, and specificity of fluorescent staining. In our experiments, we used HeLa cells that are characterized by relatively big dimensions and distinct morphology of the studied organelles. We were able to efficiently separate the nanoparticle-positive and endosomal pixels from the background pixels using the selected intensity thresholds (see Fig. 3C). Identification of ER-positive pixels was less efficient due to complex shape of this organelle (mesh-like network) and diameter of the tubules that was similar to the pixel dimensions (50–100 nm [17] and 100–200 nm, respectively). Therefore, the applied pixel-based analysis apparently overestimated the nanoparticle accumulation in ER (Fig. 4B). It is expected that robustness of ‘IntraCell’ algorithm will be higher in organelles with simple shape and distinct morphology (e.g., nucleus and Golgi) and in big adherent cells, as compared to small round cells (e.g., HeLa vs. lymphocytes or dendritic cells). Analysis of drug/DDS localization in non-continuous organelles can be potentially improved by enhancement of the experimental images, e.g., by deconvolution.

4.2. Analysis of intracellular targeting of the developed DDSs using ‘IntraCell’ plugin

In this study we applied the developed ‘IntraCell’ plugin to investigate intracellular targeting of novel formulation comprised of PLGA nanoparticles decorated with peptidic targeting residues. The choice of the formulation was based on several considerations/requirements. The size (diameter) of the particles in the ~200–500 nm range was chosen since such nanoparticles are big enough to efficiently encapsulate the cargo (water-soluble antigenic peptide and fluorescent marker) and gradually release it over several days, and are small enough to be endocytosed by the cells, and to be moved by the intracellular trafficking mechanisms. The choice of the polymer (PLGA) was based on presence of carboxylic groups in its chemical structure that are suitable for conjugation of the targeting residues (using a well-studied carbodiimide chemistry in aqueous buffer) and previous reports on enhanced cytosolic delivery of the PLGA nanoparticles following endocytosis (‘endosomal escape’ [18]). Although we were not able to determine the efficiency of the individual conjugation steps, we estimate that the applied conjugation approach resulted in decoration of nanoparticle surface by at least dozens of targeting or control residues, as obtained in other studies that used similar conjugation approaches [19,20].

Decoration of the nanoparticles with peptidic residues affected their uptake and intracellular trafficking in HeLa cells. Control peptidic-conjugated nanoparticles showed certain tendency for accumulation in the ER and early endosomes, apparently due to the cationic nature (2 lysine residues) of the decorating peptide. Targeting peptidic-conjugated nanoparticles, accumulated to a higher extent in the ER and early endosomes (significantly higher, as compared to the unconjugated nanoparticles). It appears, therefore, that this targeting peptide was identified as ER-targeting signal by the intracellular trafficking mechanisms in HeLa cells and that these mechanisms can handle cargo (nanoparticles) of the size comparable to some intracellular vesicles (hundreds of nanometers in diameter). We conclude that decoration of nanoparticles with peptidic residues affects their intracellular localization and trafficking and can be potentially used for intracellularly-targeted drug delivery.

In this study we used the developed plugin to assess the nanoparticle uptake and intracellular localization in the model cells with ‘convenient’ size and morphology (HeLa cell line, see above). In a separate experimental set, we used ‘IntraCell’ plugin and immunological assays to assess the uptake of the developed formulations in the antigen-presenting cells and the effect of intracellular targeting of the antigenic peptide on efficiency of its cross-presentation in these cells [21]. Subsequently, in vivo anti-cancer vaccination efficiency of the developed formulations should be assessed in animals with established tumors and in tumor protection assays. We expect that the developed formulations will be endocytosed by the antigen-presenting cells following vaccination, and that gradual release of the antigenic peptide in the ER and endosomal compartments (the major sites of antigen cross-presentation) will result in prolonged cross-presentation of the antigen by the antigen-presenting cells leading to enhanced activation of cytotoxic T lymphocytes directed against the tumor cells [22,23].

In conclusion, intracellularly-targeted drug delivery is a promising new approach for enhancing and controlling the drug pharmacological activities. Studies that quantitatively assess the mechanisms, barriers, and efficiency of intracellular drug delivery are required to determine the therapeutic potential of intracellular targeting of nano-delivery systems. In this study we report development and application of ‘IntraCell’ plugin that enables quantitative assessment of efficiency of intracellular drug delivery. Using
this plugin, we revealed that decoration of nanoparticles with specific targeting residues can affect their intracellular fate and lead to their preferential accumulation within an organelle of interest. ‘IntraCell’ plugin is an useful tool for quantitative assessment of efficiency of uptake and intracellular drug targeting. In combination with other experimental approaches, it will be useful for development of intracellularly-targeted formulations with enhanced and controlled drug pharmacological activities such as delivery of antigenic peptides for anticancer vaccination and for other applications.

Acknowledgments

This study was supported by the New Faculty Member Grant (Ben-Gurion University of the Negev) and Prof. Yannai Tabb Cancer Research Foundation Grant to D.S. We thank Mrs. Mazal Rubin for technical assistance, Dr. Ayelet David, Prof. Sofia Schreiber-Avissar (all from Dept. of Pharmacology, Ben-Gurion University), and Prof. Smadar Cohen (Dept. of Biotechnology Engineering, Ben-Gurion University) for reagents and research tools.

References