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Optimizing siRNA efficacy through alteration in the target cell-adhesion substrate interaction

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Abstract

The clinical potential of siRNA based therapeutics remains hindered by the challenge of delivering enough siRNA into the cytoplasm to yield a clinically relevant effect. Although much research has focused on optimizing delivery vehicles for this class of molecules, considerably less is known about the microenvironmental influences on the response of target cells to siRNA. The substrate to which cells adhere is one component of the microenvironment that can modulate cellular behavior. Here, we tested the hypothesis that modulating the properties of cellular adhesion substrates can alter siRNA efficacy. Specifically, cationic lipid complexed siRNA particles were applied to U251 cells seeded on alginate hydrogel surfaces with systematic variation in elastic modulus and integrin ligand RGD density. These experiments revealed no change in siRNA mediated eGFP knockdown over the elastic modulus range tested (53 to 133 kPa). However, an eight-fold increase in RGD content of the alginate growth substrate resulted in an increase in siRNA knockdown efficacy from $25 \pm 12\%$ to $52 \pm 10\%$, a more than two fold increase in silencing. Our results identify control of the cell-adhesion substrate interaction as a modulator of siRNA protein silencing efficacy.

Keywords

siRNA; adhesion; hydrogel; modulus; RGD

Introduction

SiRNA therapeutics present a potentially revolutionary class of clinical interventions. However, like other biomacromolecule therapeutics, delivery of siRNA remains a major barrier to its use. Extensive studies have examined the utility of siRNA delivery vectors for improved siRNA efficacy[1–2]. In contrast, less is known regarding the influence of cellular adhesion substrates on siRNA mediated protein silencing.

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The adhesion substrate on which cells grow has been previously shown to alter the delivery of other, non-siRNA, nucleic acids[3–6]. Early experiments demonstrated that modulation of ECM substrate composition can alter exogenous plasmid DNA expression levels. This concept has been further elucidated in extracellular matrix models composed of alginate hydrogels where substrate properties can be controlled independently. Specifically, the stiffness or elastic modulus can be controlled via ionic calcium crosslinker concentration and the extent of RGD hydrogel modification can be controlled to alter the density of integrin adhesion ligands[7]. Using this system, increased RGD density and increased mechanical stiffness of alginate hydrogels were shown to independently increase the exogenous gene expression of adherent pre-osteoblast cells transfected with polyethylenamine complexed DNA. Increases in gene expression correlated with increased cellular proliferation on these adhesion substrates[8–9]. This suggested that nuclear access by the plasmid during mitotic nuclear membrane breakdown facilitated plasmid expression.

Although siRNA is a nucleic acid, its mechanism of action does not require nuclear entry[10]. Thus, it is of interest to explore how the same adhesion substrate factors that influence DNA expression may also alter siRNA mediated protein silencing. We hypothesized that modifying the properties of cellular adhesion substrates could change siRNA efficacy. Towards evaluation of this hypothesis, alginate cellular adhesion substrates were generated with alteration in two variables: adhesion substrate RGD ligand density and substrate stiffness. These variables were chosen because they were previously shown to modulate the delivery efficacy of DNA[8–9]. Subsequently, the growth characteristics of stably transfected eGFP U251 (human glioma) cells seeded on the different alginate substrates were characterized. Then, siRNA mediated knockdown of the eGFP protein was used for cells on the different alginate substrates to determine siRNA efficacy. U251 cells were used because we were interested in potential factors that alter siRNA silencing in therapeutically relevant glioma model-gliomas are a prime target for siRNA delivery because they are highly invasive, malignant tumors with poor prognoses. Thus, this work uses cells that would be a potential therapeutic target for siRNA as a model system.

Materials and Methods

Materials

Dulbecco's Modified Eagles' medium (DMEM), fetal bovine serum, penicillin, streptomycin, Lipofectamine 2000, Trypsin/EDTA and Optimem I medium was obtained from Invitrogen (Carlsbad, CA). N-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Pierce Chemical (Rockford, IL). Geneticin, MES Buffer, activated charcoal and EDTA were obtained from Sigma Aldrich (St. Louis, MO). MWCO 2500 Dialysis Membranes were purchased from Fisher Scientific (Pittsburg, PA). Ultrapure MVG alginate was obtained from FMC Biopolymer (Princeton, NJ). RGD peptide G-4RGDASSKY-OH was supplied by Peptides International (Louisville, KY). SiRNA was purchased from Integrated DNA Technologies (Coralville, IA). Alexafluor488 labeled siRNA was purchased from Qiagen (Valencia, CA).

Methods

Alginate Hydrogel Synthesis—The synthesis of RGD modified alginate hydrogels and their mechanical characterization has been previously described[11–12]. In brief, alginate was covalently conjugated to RGD peptides via EDC/SulfoNHS coupling to yield differing amounts of RGD per alginate polymer (degree of substitution). For example, a gel designated DS 2 has an average of 2 RGD ligands per alginate macromolecule. Hydrogels were subsequently formed from RGD-modified alginate using calcium cross-linking, and the elastic moduli of gels was adjusted by altering the concentration of calcium used to form the

gels. Four types of ionically cross-linked alginate hydrogels were generated as cellular adhesion substrates, summarized in Table 1.

Cell Culture—Human malignant glioma U251 cells stably transfected with the pEGFP-c1 cDNA plasmid via electroporation were a generous gift from Yong Choi and John Park (NINDS, Bethesda, MD). In order to achieve a narrow range of eGFP expression levels, clonal populations of eGFP expressing U251 cells were expanded via limited dilution cloning. Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 µg/ml penicillin and 100 IU streptomycin. 1 mg/ml geneticin was also added to the cell media in order to maintain eGFP expression, since the pEGFP-c1 plasmid includes a geneticin resistance cassette. Trypsin/EDTA was used to passage the cells biweekly. They were maintained at 37°C humidified 5% CO₂ incubator.

Characterization of Cell Growth, Surface Area Distribution and Cell Number—Cylindrical alginate gels were prepared as described above with a thickness of 1mm and 10 mm diameter. They were incubated in 0.5 ml of complete DMEM growth medium overnight at 37 degrees Celsius and 5% CO₂ prior to transfer into wells of a 24 well plate. A clonal population of eGFP U251 cells was then seeded onto each alginate gel at 6×10^4 cells/0.5ml of growth media. For fluorescent imaging, cells were visualized with a Nikon Eclipse E800 fluorescent microscope and images were taken with a Nikon camera attachment. ImageJ was used to characterize both surface area distributions of the cells and their number from fluorescent images (NIH, Bethesda, MD).

Evaluation of siRNA Mediated Knockdown of eGFP and siRNA uptake—Clonal eGFP U251 cells were seeded and grown as described above, except that the media used was antibiotic free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. After allowing the cells to incubate at 37 degrees Celsius and 5% CO₂, siRNA complexed to Lipofectamine2000 was added at various concentrations to each well of the 24 well plate. Each well contained a single alginate gel with adherent eGFP U251 cells. The siRNA sequence used to silence eGFP is as follows: sense-GCAGCACGACUUCUUAAGTT, antisense-CUUGAAGAAGUCGUGCUGCTT. The scrambled siRNA control sequence is as follows: sense-GUGACAUCGCGACCACUUATT, anti-sense-UAAGUGGUCGCGAUGUCACTT. Medium within each well was aspirated, and the siRNA-Lipofectamine2000 complexes suspended in 100 µL of OptiMEM I were applied to each well. After 4 h, the medium containing the siRNA-Lipofectamine2000 complexes was aspirated and replaced with 500 µL of complete growth DMEM medium without siRNA-Lipofectamine2000 complexes. The cells were then allowed to further incubate at 37 degrees Celsius and 5% CO₂ for 72 hrs. At this time, the gels and their adherent cells were removed from their wells and placed in 1 ml of 50mM EDTA in PBS for approximately 15 min until dissolved. The cells were then pelleted, resuspended in 300 µL D-PBS, and filtered through a cell strainer into FACS tubes. Samples were analyzed with the LSR Fontessa (BD Biosciences, San Jose, CA) flow cytometer and associated FACSDiva software. Events were recorded and gated based on FSC/SSC for viable cells. Further analysis and graph plots were generated with FlowJo V.6 software (TreeStar Inc., Ashland, OR).

A similar procedure was performed to assess the fluorescent AlexaFluor488 labeled siRNA uptake. However, instead of FACS analysis at 72 hrs, analysis was performed directly after wash-out of the siRNA –Lipofectamine2000 complexes on Day 1 post seeding, the initial cell seeding density was 6×10^4 cells/0.5 mL of growth media on each alginate gel and the concentration of AlexaFluor488 labeled siRNA used was 50 nM. siRNA uptake was

determined by comparison of mean fluorescent intensity at the 488 wavelength for cells with siRNA applied and untreated control cells.

Statistical Analysis—Statistical analysis for significance via one-way analysis of variance (ANOVA) with post hoc Scheffe test was performed using SPSS 17.0 (SPSS, Chicago, IL). Statistical analysis with Student T tests were performed with Excel 2007 (Microsoft, Redmond, WA).

Results and Discussion

Alginate hydrogel adhesion substrate synthesis and characterization

These alginate hydrogels were systematically varied via two properties: RGD peptide density and elastic modulus (E), as shown in Table 1. RGD density was modulated via covalent conjugation to yield a ratio of RGD ligand to alginate polymer chain (degree of substitution) of 2 and 16. Prior reports have indicated that that ionically crosslinked hydrogels can lose mechanical strength over time as calcium ions that crosslink the guluronic acid (G) blocks of the alginate polymers exchange with monovalent ions in the incubating media[13]. Determination of the elastic modulus over the time courses used in the experiments of this study demonstrated that this phenomenon was too gradual to dramatically affect the stiffnesses of the gels (Figure 1A). Furthermore, covalent modification with RGD did not appreciably alter the hydrogel's mechanical properties (Figure 1B).

Characterization of eGFP U251 cell growth upon alginate substrates

Prior to determining the effects of alginate adhesion substrates on siRNA efficacy, the growth of U251 cells upon these surfaces was characterized. Previous work established the necessity of RGD ligands for cellular adherence to alginate substrates, since alginate alone does not allow for mammalian cell adhesion[14]. Furthermore, there is often a threshold limit of RGD grafting, below which cells do not adhere to the gel. This lower limit is cell type dependent[14–15].

Initial characterization of cell morphology, proliferation and surface area was performed for the RGD modified alginate substrate at DS 2 and DS 16, with either an elastic modulus of 53 kPa or 133 kPa. Cells were able to adhere to both DS 2 and DS 16 alginates of both stiffnesses tested (Fig. 2). General cellular morphology did not vary greatly between the different alginate substrates. However, in comparison to cells growth on tissue culture polystyrene (TCPS), there were a higher proportion of cells with an elongated appearance on the alginate gels. The decrease in elongated cells on the TCPS surfaces may be due to a higher density of adsorbed adhesion molecules on TCPS than on the alginate surfaces or to the stiffer substrate.

In addition to qualitative observations of cell shape, cell surface area was also quantified for each of the four different gels and TCPS. These readings were performed since alterations in cellular surface area might change the opportunities for siRNA particle internalization, and thus, the efficacy of delivery. Slight differences in cellular surface area were seen for cells grown on the four different alginate substrates (Fig. 3), although all four demonstrated an approximately Gaussian distribution of surface area measurements (data not shown). The average surface area for the 53 kPa and 133 kPa alginate gels with DS2 were 338 and 316 μm^2 , respectively. Cells growing on the DS16 substrates possessed slightly larger average surface areas of 390 and 440 μm^2 , for 53 kPa and 133 kPa, respectively. Finally, cells grown on TCPS had the largest average surface area of 561 μm^2 .

Previous work has indicated that the ability of an adhesion substrate to increase cellular proliferation improved the delivery efficacy of DNA/PEI complexes to cells, so the proliferation of U251 cells was approximated over four days by cell number counts[8–9]. Although cell number is an aggregate reading that conflates rates of cell proliferation and cell death, cells grown over this time period were supplied with adequate nutrients and were subconfluent, making it unlikely that cell death contributed substantially to deplete cell number counts. The total number of cells was highest on gels with the greatest RGD density (DS 16) and for the stiffer gels with DS 2 (Fig. 4A). However, when the total cell number was normalized to the number of cells present at day 1, there was no significant differences between the substrates (Fig. 4B). This is in contrast to what has been observed with other cell types including myoblasts and hepatocytes, where increased stiffness and RGD density encourage increased proliferation[14–16]. It is possible that this difference from previous findings may result from differences in cell types. Specifically, the human glioma U251 cells used in these experiments may be under reduced proliferative control by the adhesion substrate, in contrast to the non-neoplastic cell lines used previously. Alternatively, the U251 may not be responsive to the range of elastic moduli used in this study.

SiRNA gene silencing efficacy on alginate substrates

After characterization of the cellular spreading and proliferation of eGFP U251 cells on the alginate adhesion substrates, the effect of these substrates on siRNA silencing efficacy was assessed. To accomplish this, siRNA with a sequence designed to silence eGFP was applied following complexation with Lipofectamine 2000, at concentrations of 8.25 nM and 33 nM. It is that eGFP protein levels will be a direct reflection of eGFP mRNA levels, and that effective presumed siRNA silencing results in a decrease in both eGFP mRNA and protein, although only eGFP protein levels were quantified. On TCPS, U251 cells exposed to 8.25 nM had a 44 ± 3 % decrease in eGFP expression (Fig. 5). This silencing was due to sequence specific siRNA mediated knockdown, since a scrambled siRNA control showed no significant siRNA knockdown at 8.25 nM (Supplementary Figure 1). However, the growth of the same cells on alginate adhesion substrates completely eliminated the ability of siRNA at this concentration to exert a significant knockdown effect (Fig. 5). At a higher concentration of 33 nM siRNA, siRNA application to cells adhered on TCPS resulted in an 80 ± 2 % knockdown in eGFP expression after three days (Fig. 5). Again, this was a sequence specific effect, since 33 nM of sequence scrambled siRNA control showed only a 9% decrease in eGFP expression (Supplementary Figure 1). In contrast to results with the lower siRNA concentration, siRNA knockdown was observed for cells adherent to the alginate gels, although to significantly decreased levels than that for cells on the TCPS substrate.

The highest knockdown seen for U251 cells on the alginate substrates was 52 ± 12 % on the alginate gel of 133 kPa and DS 16. This gel had the highest mechanical rigidity and highest RGD content of all the adhesion substrates tested. The other adhesion substrate with DS 16, but a reduced stiffness (53 kPa) demonstrated a similar 47 ± 14 % degree of knockdown. In contrast, for the same elastic modulus, reducing the DS from 16 to 2 also resulted in a significant decrease in knockdown efficacy. For the softer gels, an eightfold reduction in RGD content reduced the knockdown efficacy of the anti-eGFP siRNA by 52% to 25 ± 10 %. A similar result was observed for the stiffer gels, as in this case, the knockdown efficacy was reduced by 27% to 34 ± 6 % (Fig. 5).

These results indicate that for the range of stiffness values tested (53 to 133 kPa), the efficacy of siRNA mediated knockdown in U251 cells is not significantly impacted. This finding is in contrast to results obtained previously for DNA delivery, whereby changes in the elastic modulus of the alginate gels resulted in significant alterations in exogenously introduced DNA expression in pre-osteoblasts[9]. Although it is not possible to definitely

identify the mechanistic reason for this difference from the present experiments, one possibility may be cell line differences in the range of elastic moduli that elicit a biological response. The modulus range of most organs and tissues ranges from 0.1 to 100 kPa, and the moduli of the alginate substrates used in the studies fall within this range[17]. For example, U251 cells may be less sensitive to adhesion substrates in the range of 53 to 133 kPa, and require testing at an increased range of substrate stiffness to reveal alteration in siRNA efficacy. This is consistent with the U251 cell line's origin from a human neoplasm, since cancer cells are generally less responsive to extracellular matrix cues than normal tissue cells. In regard to TCPS substrates, it is possible that the higher stiffness of the TCPS accounts for the increased siRNA silencing efficacy. Yet, because of the different composition and surface properties of TCPS compared to RGD modified alginate, one cannot exclude that properties of TCPS beyond simple stiffness are altering the siRNA efficacy.

In contrast to the impact of gel stiffness, the RGD content of the substrates did have a significant effect on the ability of siRNA to mediate protein knockdown. Specifically, increasing the RGD density within the substrate improved siRNA knockdown, as demonstrated between the significant increase in siRNA efficacy for cells grown on 133 kPa and DS16 alginate gels compared to 133 kPa and DS2 alginate gels. These results are unlikely to be due to changes in the ratio of cell number to siRNA concentration, because the initial seeding density of U251 cells on the DS2 alginate gels was lower than on the DS16 gels (Fig. 4). Thus, the amount of siRNA available per cell is actually higher for the DS2 gels compared to DS16. In addition, other significant differences between cell surface area and morphology were not found between the different alginate substrates that could account for the RGD-mediated protein silencing effects observed. In more detail, siRNA was applied to the cells at Day 1 post-seeding, and at this time, there was only one significant difference between cellular surface area when cells grown on the different alginates were compared. However, there was not a significant difference in the surface area between the cells grown on 133 kPa/DS 2 and 133 kPa/DS 16 gels at the time of siRNA application on post-seeding Day 1, and it was these substrates that demonstrated a significant difference in siRNA knockdown. Thus, it is unlikely that surface areas on the different substrates accounts for the differences in siRNA knockdown efficacy amongst the various alginate substrates. Furthermore, although the surface area of the cells grown on TCPS was significantly different from all of the alginate substrates at Day 1 post-seeding, normalizing the knockdown percentage effect for TCPS and the different alginate substrates by surface area at Day 1 post-seeding results in an elimination of the significant difference of siRNA knockdown between TCPS and some, but not all, of the alginate gels. This suggests that changes cell surface area alone may partially correlate with some of the differences in knockdown seen for cells grown on TCPS in comparison to alginate substrates. However, because significant differences in siRNA efficacy remain after surface area normalization, this factor alone does not account for all of the difference in substrate effects.

To explore the possible mechanisms of the increased siRNA knockdown efficacy, cells grown on the different substrates were analyzed for siRNA uptake differences. SiRNA uptake was quantified by measuring the fold increase in cellular fluorescence at 488 nm for cells exposed to AF488 labeled siRNA at 1 Day Post-seeding on the different substrates as compared to a non-siRNA treated control. Cells growing on alginate substrates with higher RGD density demonstrated statistically significant increases in siRNA uptake (Figure 6A). Furthermore, the significant difference in siRNA silencing efficacy seen between the 133 kPa/DS 2 gel and the 133 kPa/DS 16 gel is eliminated by normalization for siRNA uptake (Figure 6B). Our presented results suggest a correlation between increased siRNA uptake on alginate substrates with RGD density. However, it does not preclude the other effects of

increased RGD density on siRNA silencing including increased activity of endogenous siRNA silencing protein effector complexes and /or increased intracellular siRNA stability.

Conclusions

The results of this study demonstrate that adhesion substrate properties can significantly affect the efficacy of exogenously delivered siRNA. Human glioma U251 cells seeded on alginate hydrogels of varying stiffness and RGD density did not demonstrate qualitative differences in cell shape nor quantitative differences in surface area or cell number over time. However, increasing the alginate RGD density significantly increased the efficacy of siRNA knockdown when applied at 33 nM, while changes in elastic modulus did not alter siRNA efficacy. The increase in siRNA knockdown efficacy at higher RGD density occurred despite the fact that a lower effective siRNA concentration was available per cell in this condition. A lower siRNA concentration (8.25 nM) did not result in siRNA mediated protein expression silencing for any of the alginate adhesion substrates tested. For both concentrations of siRNA tested, cells grown on TCPS demonstrated considerably more siRNA mediated protein knockdown than those grown on any of the alginate substrates. These results support the conclusion that adhesion substrate properties can change the cellular response to siRNA. Because of the interest in siRNA as a therapeutic, such findings have important implications for pre-clinical testing of siRNA based therapeutics and for siRNA applications within the *in vivo* setting, in which cells grow in diverse extracellular environments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(A) The elastic modulus of 2% by weight alginate hydrogels with 50 mM ($\square$) and 100 mM ($\blacksquare$) calcium crosslinker were tested over a four day time course. Values shown represent the mean and error bars represent the standard deviation of three experiments. After four days, gels synthesized with 50 mM and 100 mM Ca^{++} had elastic moduli of $53 \pm 23\text{kPa}$ and $133 \pm 31\text{kPa}$, respectively. (B) Comparison of the mechanical properties of alginate hydrogels without and with a high degree of RGD modification. The calcium cross-linker concentration was held constant at 50 mM (53 kPa elastic modulus) for hydrogels formed from 2.0% weight percent alginate without RGD modification (light gray bars) and with DS16 RGD modification (dark gray bars). Values represent the mean and error bars represent the standard deviation of three experiments. No statistically significant differences between the mean elastic values of alginate hydrogels with and without RGD modification were found.

Figure 2.
Morphology of eGFP cells adherent to substrates of varying RGD density and varying stiffness one day post seeding. Bar represents 100 μm .

Figure 3.

Surface areas of cells adherent to different types of alginate gels over time. Surface area shown for U251 cells grown on 53 kPa DS 2 alginate gels (black), 133kPa DS 2 alginate gels (dark gray), 53 kPa DS16 alginate gels (light gray), 133 kPa DS 16 alginate gels (white) and TCPS (striped) at either one, two or four days post seeding. Measurements were taken for cells measured in six 0.58 mm² fields on each gel. Values represent mean cellular surface area and error bars represent the standard deviation among average cellular area of each 0.58 mm² field. Asterisks indicate $p < 0.05$ by ANOVA with post-hoc tests.

A.

B.

Figure 4.

Cellular number for growth on alginate substrates of varying RGD density and stiffness over a four day period. (A) Cell number shown for U251 cells adherent to 53 kPa DS 2 alginate gels (black), 133 kPa DS 2 alginate gels (dark gray), 53 kPa DS16 alginate gels (light gray), 133 kPa DS 16 alginate gels (white) and TCPS (striped) at either one, two or four days post seeding. (B) Data from part A normalized to number of cells present in each condition on gels at day 1. Values represent mean cell number and error bars represent the standard deviation for four randomly chosen 0.58 mm² fields. ANOVA with post-hoc tests revealed no significant differences between the different cell substrates at Day 1 Post-Seeding and when compared at the same day post seeding with normalization to cell number at Day 1.

Figure 5.

Relative eGFP expression in U251 cells on alginate adhesion substrates differing in RGD density and mechanical stiffness after application of 8.25nM (light gray) and 33 nM (dark gray) of siRNA. eGFP expression was assayed via FACS three days after siRNA application on at least 50 cells per treatment. Values represent the mean and error bars represent the standard deviation of three replicate experiments. Asterisks indicate $p < 0.05$ in a student t test either for relationships indicated via line or in comparison to all other treatments of the same siRNA concentration.

Figure 6.

(A) siRNA uptake, normalized to a non-siRNA treated control, after a 4 h incubation with 50 nM AF488 labeled siRNA in U251 cells 1 day post-seeding on different growth substrates. Uptake was assayed via FACS immediately after the 4 h uptake period for fluorescent siRNA on at least 50 cells per treatment. Values represent the mean and error bars represent the standard deviation of three replicate experiments. Asterisks indicate $p < 0.05$ in a student t test either for relationships indicated via line or in comparison to all other treatments of the same siRNA concentration.

(B) eGFP expression in U251 cells on alginate adhesion substrates differing in RGD density and mechanical stiffness after application of 8.25nM (light gray) and 33 nM (dark gray) of siRNA, normalized for fold increase in mean fluorescent intensity at 488 nm wavelength one day after seeding. eGFP expression was assayed via FACS three days after siRNA application on at least 50 cells per treatment. Values represent the mean and error bars represent the standard deviation of three replicate experiments. No significant differences between growth substrates when compared for the same siRNA treatment concentration was found with ANOVA post hoc tests.

Table 1

Four types of alginate hydrogels were synthesized with systematic variation in RGD density and elastic modulus.

Degree of Substitution (DS)	Ca ²⁺ Concentration (mM)	
	50	100
DS 2	DS 2, 53±23 kPa	DS 2, 133±31 kPa
DS 10	DS 10, 53±23 kPa	DS 10, 133±31 kPa