Published in final edited form as:

Biotechnol Bioeng. 2014 February ; 111(2): 396–403. doi:10.1002/bit.25097.

Multiwell stiffness assay for the study of cell responsiveness to cytotoxic drugs

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Abstract

It is now well understood that the cell microenvironment, including the surrounding matrix, profoundly affects cell fate. This is especially true for solid tumors where, for example, matrix stiffness is believed to be an important factor in tumorigenesis. Our hypothesis is that since matrix stiffness affects cell fate, it may also be important in drug resistance. To test this hypothesis, we designed and built a multiwell polyacrylamide (PA) gel-based stiffness assay, in which the gels were coated with collagen in order to facilitate cell attachment and proliferation. This PA-based assay was used to examine the effect of stiffness on cultured cell responsiveness to cytotoxic drugs. In particular, we tested multiple cancer cell lines and their susceptibility to paclitaxel, a microtubule-targeting agent. By assessing cell proliferation, morphology, and the IC₅₀ of the drug, we were able to establish that the stiffness affects responsiveness to cytotoxic drugs in a cell dependent manner.

Keywords

substrate stiffness; polyacrylamide; cancer; paclitaxel (Taxol®); drug resistance

INTRODUCTION

Current research suggests that cancer cells grown on plastic tissue culture dishes, as opposed to biomaterial-based matrices, have different susceptibilities to cytotoxic chemotherapy drugs (Minchinton and Tannock 2006), yet the underlying reasons are largely unknown. One of the key factors that affects cell fate as well as cells' responsiveness to cytotoxic drugs is the stiffness of the substrate upon or within which the cells rest, which also has been linked to the progression of malignancy (Discher et al. 2005; Levental et al. 2009; Paszek et al. 2005). Despite the possible implications of material stiffness for cell fate, the study of cells on substrates of different stiffness has rarely been performed in a "high-throughput" multiwell plate format. Consequently, systematic studies relating matrix stiffness to cancer cells' responsiveness to cytotoxic chemotherapy drugs are rare.

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The focus of this work has been the development of a multiwell stiffness assay as a drug screening platform and subsequent validation of the assay with a small scale drug screen. Soft PA hydrogels are the material of choice for exploring the effect of stiffness on cell fate due to their viscoelastic properties, which closely emulate those of the extracellular matrix over a wide, physiologically relevant range of elastic modulus, namely 0.1 – 300 kPa (Pelham and Wang 1997). However, existing methods to fabricate PA gels for cell culture are labor intensive and typically produce assay materials in small batches. Several groups have attempted to overcome this limitation by creating PA gels in a higher-throughput format. Semler *et al.* made 1 mm thick sheets of PA gels which were subsequently cut into pieces and placed into 96-well plates (Semler et al. 2005). This method, though, is limited to stiffer gels, since gels whose moduli are less than approximately 1 kPa are too soft and sticky to manipulate. Mih *et al.* overcame this hindrance by pouring the gel solutions directly into functionalized glass-bottom 96-well plates and forming gels of a desired thickness by using a custom coverglass array (Mih et al. 2011). Even though the authors were able to achieve negligible variation in gel thickness with this technique, slight distortions in gel uniformity did occur within polystyrene wells of each plate. An additional disadvantage of this method, as well as with other more sophisticated microfabrication approaches that use flexible post arrays to manufacture surfaces of varying stiffness (Fu et al. 2010), is that it requires specialized manufacturing procedures that are not immediately accessible to many laboratories.

Here, we describe an assay which, by utilizing a transferable plastic film as a structural support for the gels, is an improvement over existing methods in several aspects: it is quick to manufacture in a multiwell plate format; it robustly produces homogenous gels of various stiffness; and, it requires only materials that are commercially available. We validated the utility of our assay by systematically testing the stiffness dependence of several cancer cell lines in terms of proliferative capacity, cell spreading, and drug responsiveness. Our drug screening test indicated that substrate stiffness can affect the cancer cell response to cytotoxic drugs in a cell type-dependent manner, which may have implications for designing more predictive drug screening platforms.

MATERIALS AND METHODS

Materials

40% Acrylamide aqueous solution and 2% bisacrylamide solution were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Irgacure 2959 was obtained from BASF Corporation (Florham Park, NJ). Red fluorescent latex beads were purchased from Molecular Probes (Grand Island, NY). GelBond PAG film was purchased from BioWhittaker Molecular Applications (Rockland, ME). Square-well 96-well plates were purchased from Matrical Biosciences (Spokane, WA). Polydimethylsiloxane (PDMS), under the trade name Sylgard 184 Silicone Elastomer, was purchased from Dow Corning Corporation (Midland, MI). *N*-Sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino) hexanoate (sulfo-SANPAH) was purchased from Thermo Scientific (Rockford, IL). Collagen Type I (rat tail, 3.68 mg/ml) was purchased from BD Biosciences (Bedford, MA). Cy5 was purchased from GE Healthcare (Piscataway, NJ). HepG2 and HeLa cells were obtained from ATCC (Manassas,

VA); MDA-MB-231, MCF-7, SW620, HT29, 786-0, ACHN, and PC3 cells were obtained from the National Cancer Institute (NCI) Drug Screening Cell Bank. SY5Y cell line was a kind gift from Dr. Carol Thiele, NCI/NIH. RPMI 1640 medium was obtained from Cell-Gro (Manassas, VA), Fetal Bovine Serum from Thermo Scientific (Waltham, MA), and trypsin EDTA from Life Technologies (Carlsbad, CA). CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI). Paclitaxel (Taxol®), dimethyl sulfoxide (DMSO), sodium azide, and hydrochloric acid were purchased from Sigma (St Louis, MO).

Gel preparation and multiwell stiffness assay assembly

Polyacrylamide (PA) gels of desired stiffness were prepared by mixing acrylamide and bisacrylamide crosslinker to a desired final concentration (Table 1) and assembled in a 96-well plate format (Figure 1). To prepare the gels, Irgacure 2959 at a final concentration of 0.1% w/v was added to all gel solutions. A 500 μ l aliquot of a given gel solution was pipetted onto the hydrophilic side of a GelBond sheet. A second GelBond sheet, hydrophobic side down, was placed over the gel solution. Parafilm strips were used as separators to define the thickness of the gel. A glass plate was positioned on top of the GelBond/gel “sandwich”, to assure uniform distribution of the gel solution onto the GelBond sheet. The gels were polymerized under UV (365 nm, Black-Ray UV Bench Lamp, UVP, Upland, CA) for 10 min, after which the glass was removed and the top GelBond sheet was peeled off. The GelBond/gel constructs were left to dry overnight at room temperature and then cut into squares having dimensions equal to those of the well area of square 96-well plates (6.4×6.4 mm). To assemble the assay, the GelBond/gel squares were placed, gel side up, into the wells of the 96-well plate on top of a PDMS droplet. The PDMS acted as a biocompatible glue attaching the gels firmly to the bottom of the wells. The multiwell plate was then left in the incubator for 4 h to allow the PDMS to solidify.

AFM measurements

The Young’s Modulus (E) of the polyacrylamide gels was measured by AFM, using a Bioscope Catalyst® NanoScope® V device (Bruker, Santa Barbara, CA) attached to an inverted optical microscope (IX71, Olympus, Japan). Polyacrylamide gels were indented with a V-shaped cantilever (MSCT, pyramidal tipped, nominal $k = 0.01$ - 0.03 N/m; Bruker) whose spring constant was calibrated by the thermal fluctuations method (Butt and Jaschke 1995; Hutter 1993). The relationship between the photodiode signal and the cantilever deflection was computed from the slope of the force displacement curve obtained at a bare region of the coverslip (without gel sample). The force (F) on the cantilever was computed using Hooke’s law ($F = kd$, where d is the cantilever deflection).

We probed five different regions for every gel. At every region, we acquired five force–displacement curves (F – z , z being the displacement of the piezotranslator) while the piezotranslator was ramped forward and backward at constant speed (1 Hz, 5 μ m amplitude, ~ 1 μ m maximum indentation). Force-indentation data were analysed with the four-sided pyramidal indenter model (Rico et al. 2005):

$$F = \frac{3 \tan \theta}{4(1 - \nu^2)} \delta^2,$$

where E is the Young's modulus, ν is the Poisson's ratio, θ is the semi-included angle of the pyramidal indenter, and δ is the indentation depth. The parameter ν is assumed to be 0.5 (the water-filled hydrogel essentially is incompressible), and the indentation depth is calculated as $\delta = z - z_0 - d$, where z_0 is the tip-gel contact point. E and z_0 were estimated by least-squares fit of this equation to the F - z curve recorded on each gel point (Alcaraz et al. 2003).

Collagen coating

Collagen coating was applied to all gel types to provide cell attachment sites to the otherwise inert polyacrylamide gels. To apply the collagen coating, the gels were first derivatized with Sulfo-SANPAH dissolved in DMSO:PBS at a ratio of 4:96. Briefly, to prepare the sulfo-SANPAH solution, the reagent was first dissolved in DMSO at 10% w/v and stored at -80°C in 20 μl aliquots until further use. Each aliquot was thawed and diluted to 0.5% w/v in deionized water immediately before use. This was then placed on top of the gels at 50 μl /well and activated by exposure to high intensity UV light for 5 min. The unreacted crosslinker was removed by a double wash with PBS, after which 50 μl of 0.2 mg/ml collagen Type I was added to each well and allowed to react for at least 2 h at room temperature. The gels were sterilized under UV for 2 h prior to cell seeding.

Fluorescent collagen was used to confirm collagen coating uniformity of the PA gel surface. For fluorescent labeling, collagen was diluted in 0.1 M Na_2CO_3 /0.5 M NaCl buffer of pH 9.3 to a final concentration of 1 mg/ml. The collagen was then reacted with Cy5 for 30 min at 4°C under constant stirring, following the manufacturer's procedures. Unreacted dye was removed by dialysis in 2 mM HCl with three changes of dialysis buffer. Final collagen concentration was measured by circular dichroism (J810, Jasco, Easton, MD). Fluorescent collagen was stored in 2 mM HCl at 4°C until further use. To attain fluorescent images of the gels as well as the collagen coating, fluorescent latex beads were added to the PA gel solution prior to gelation. Cross-sectional fluorescent images of the resulting gels were obtained with Zeiss LSM 510 META microscope.

Cell culture and imaging

Cancer cell lines of different origin were used (Table 2). All cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and incubated in a humidified incubator at 37°C and 5% CO_2 . Cells were harvested by a 5 min exposure to trypsin/EDTA and plated onto a 96-well stiffness assay plate at 10^4 cells/well. Cells were imaged under a phase contrast on an EVOS microscope (AMG, Mill Creek, WA), using a $10\times$ or $20\times$ objective. Cell spreading area was analyzed with the Micron EVOS proprietary software after manually tracing the boundary of each cell from phase contrast images.

Cell proliferation and adherence

To perform cell proliferation and adherence experiments, MTS assays were conducted 3-5 h post cell seeding and 72 h post seeding for each cell type. The comparative cell proliferation

for each cell type was then calculated using the MTS results at 72 h post seeding, and comparative cell adherence was calculated using the MTS results at 3-5 h post seeding. To aid comparison between cell types, the MTS results were normalized as follows: $[\text{OD}_{490} \text{ for 1 kPa gel}] / [\text{OD}_{490} \text{ for 100 kPa gel}]$. MTS was used as recommended by the manufacturer. Briefly, 20 μl of the MTS solution for every 100 μl of medium was added directly to the wells and the cells were then incubated for up to 1 h in a humidified incubator at 37°C and 5% CO_2 . Prior to absorbance measurements, 80 μl of the MTS:medium solution was transferred from each well into a well of a new 96-well plate in order to avoid background absorbance from the gels. Absorbance in the no-gel 96-well plate was measured at 490 nm (Spectra Max Plus, Molecular Devices, Sunnyvale, CA).

Drug screening

Paclitaxel, a microtubule-stabilizing agent, was the drug of choice. Cells were grown for 4 – 24 h prior to paclitaxel administration. Paclitaxel was administered at various concentrations between 0 and 200 nM. No-cell negative control and a no-growth control, where cell growth was halted by addition of sodium azide at 0.2% final concentration just prior to drug administration were also used. Viable cell numbers were evaluated after 72 hours of paclitaxel exposure using an MTS assay. Dose response curves and drug IC_{50} (50% inhibition concentration), were analyzed with GraphPad Prism software by fitting a four-parameter Hill slope to the normalized dose-response data. Percent cell viability was calculated as $(\text{OD}_{490} \text{ of cells at X nM Paclitaxel} - \text{OD}_{490} \text{ of cells at 200 nM Paclitaxel}) / (\text{OD}_{490} \text{ of cells at 0 nM Paclitaxel} - \text{OD}_{490} \text{ of cells at 200 nM Paclitaxel}) \times 100$.

Statistical analysis

The results of all experiments are the mean values ($\pm SD$) of three to eight samples per condition, performed in two - six independent experiments. Comparisons between multiple samples were performed with single factor analysis of variance (ANOVA) and comparisons between two samples were performed with two-tailed Student's *t*-test, followed by post-hoc analysis. In all cases, differences between data sets were considered significant when $p < 0.05$.

RESULTS AND DISCUSSION

Characterization of multiwell stiffness assay

PA gels of 1, 10, and 100 kPa were placed in 96-well plates as discussed in Materials and Methods and depicted on Figure 1. The gels were made on top of a GelBond plastic sheet and subsequently cut into squares that cover the bottom of the wells. This approach allowed us to scale the PA gels to a multiwell format in a robust and rapid manner, while avoiding edge gel distortions characteristic of other assays (Mih et al. 2011). In addition, it rendered the lengthy glass pretreatment steps associated with making PA gels (Pelham and Wang 1997) unnecessary. Further, once dried, gels cast on GelBond can be stored indefinitely and thus could be made in advance in large quantities. The GelBond support also allowed us to cast and easily handle very soft thin gels. Additional advantage of our approach was that by making the gels between two transparent plastic sheets, we were able to use UV crosslinking for gel formation as opposed to the standard catalyst driven crosslinking, which utilizes

ammonium persulfate and tetramethylethylenediamine (TEMED) (Yeung et al. 2005). UV crosslinking shortened the gelation time and rendered the use of the toxic TEMED unnecessary.

The use of PDMS to attach the GelBond/gel constructs to the bottom of the wells assured that all substrates remained firmly attached to the plate during vigorous washing, media changes, and other cell culture steps. One cautionary note is that when soft gels, 1 kPa or less, are made on GelBond and allowed to dry, the gel surface might occasionally appear cracked upon rehydration. This behavior is common for geometrically constrained gels of low modulus that undergo dehydration and subsequent re-swelling (Saha et al. 2010; Trujillo et al. 2008). Thus, all 1 kPa gels were visually observed before use and cracked gels were discarded. Gel stiffness was measured via AFM prior to collagen coating (Table 1). Representative force distance (FD) curves for 1, 10, and 100 kPa PA gels are shown on Supplemental Figure 1, where the closed symbols represents approach and the open symbols represent withdrawal of the cantilever. Predictably, the slope of the curve increased with increase in gel stiffness.

We used the crosslinker sulfo-SANPAH which mediates succinimide crosslinking to attain efficient uniform collagen coating (Supplemental Figure 2). It has been shown that succinimide chemistry allows the incorporation of a larger amount of protein on the gel surface compared to nonspecific associations (Sunyer et al. 2012). The presence of the collagen coating was tested via two different methods. First, we seeded SY5Y cells on non-coated and collagen-coated gels and compared the results after 24 h of cell seeding (Supplemental Figure 3). We observed uniformly distributed and well spread cells on the coated gels, while only a few rounded cells were observed on the uncoated gels. This result was expected because collagen coating is important for cell attachment and survival on the otherwise inert PA gels. Similar tests were performed for all gel types, namely 1, 10, and 100 kPa, confirming that all gel types were collagen coated. To further inspect the uniformity of the collagen coating we captured confocal images of PA gels of varying stiffness containing embedded fluorescent microspheres and coated with fluorescently labeled collagen (Figure 2). We observed that the collagen was confined to the top of the gels and uniformly distributed across the gel surface for all gel types. Our results correlate well with findings from the literature. For example, Mih *et al.*, who used chemiluminescence to test the efficiency of collagen binding to PA gels of various stiffness in the range 0.3 – 55 kPa, showed not only that all gels were uniformly coated, but also that collagen presentation could be tuned independently of stiffness by applying various amounts of collagen during the crosslinking step (Mih et al. 2011).

In addition, the gel images were used to quantify gel thickness which was found to be 130 μm for the 1 kPa gels and ~ 100 μm for the 10 kPa and 100 kPa gels. The higher thickness of the soft gels was attributed to increased swelling due to decreased bisacrylamide crosslinking. Gel thickness is an important parameter in stiffness assays because the cells are able to sense the underlying support and to respond by adjusting their rigidity, contractile forces, and spreading area. However, this sensing of the underlying surface becomes negligible when the substrate thickness is larger than a characteristic thickness given by the lateral dimension of the cell (Lin et al. 2010). For our purposes, we used a minimum gel

thickness of 100 μm because the lateral dimension for all tested cell types was below that number.

Cell growth and proliferation on multiwell stiffness assay

Various cell types have been reported to proliferate more rapidly on stiffer substrates (Klein et al. 2009; Tilghman et al. 2010; Wells 2008) where matrix stiffening has also been implicated to play a role in tumorigenesis (Levental et al. 2009; Paszek et al. 2005). The majority of the studies on the stiffness dependence of cell proliferation have been focused on a single cell type (Subramanian and Lin 2005; Yoshikawa et al. 2011). The true contribution of our assay is allowing a rapid, low-cost, and systematic testing of multiple parameters and conditions. Thus, to explore cell proliferation dependency on stiffness in a systematic manner, while validating the utility of the developed assay, we examined the proliferation of several cancer cell lines 72 h post-seeding as a function of matrix stiffness (Figure 3). Viable cell number was measured at 72 h post-seeding with an MTS assay, which measures the metabolic activity of live cells. To aid comparison between cell types, the results were normalized by the average proliferation (OD_{490}) on the 100 kPa PA gels for each cell type. The chosen cell lines represent soft solid tumors for which the native healthy tissues have stiffness comparable to the stiffness range covered by our assay (Dall et al. 1993; Levental et al. 2009; Miller et al. 2000; Myers et al. 2008; Yeh et al. 2002). Since the developed assay ultimately depends on cell-matrix interactions, all cell types were also adherent cancer cells for which the ECM microenvironment plays an important role in cell growth and proliferation (Sounni and Noel 2013).

The results shown in Figure 3 indicate that cell proliferation is affected by matrix stiffness in a cell type-dependent manner. All cell types, except SW620 and PC3, showed higher proliferative capacity on stiffer substrates. In general, we saw no correlation between the stiffness dependence of cell proliferation and the tissue of origin. For example, the two colon cancer cell lines HT29 and SW620 exhibited stiffness-dependent and stiffness-independent growth profiles, respectively. Overall, our current results correlate with trends discussed in the literature, where other researchers have shown that most cell lines are responsive to the stiffness of the substrate: for example, MDA-MB-231 cells exhibit >20 fold increase in cell numbers over 5 days on 9.6 kPa gels as opposed to 1-5 fold increase on 0.15 kPa gels (Tilghman et al. 2010) and HepG2 cells show a 12-fold higher proliferative index on 12 kPa PA gels versus 1 kPa gels (Schrader et al. 2011).

To confirm that the results shown in Figure 3 are indeed due to proliferation rather than variable cell adherence on substrates of different stiffness, we seeded the cells on the multiwell stiffness assay for 3-5 hours and measured cell metabolic activity via an MTS assay (Figure 4) but, in order to measure only the adhered cells, the media was changed prior to using the MTS assay. Our results indicate that at 3-5 hours post-seeding all cell types adhered equally well on PA gels of different stiffness. These results were expected, as cell adherence correlates with the existence of a sufficient number of integrin binding sites, influenced by the density of the collagen coating, which should be comparable for all gel types. Similar cell adhesion efficiency to collagen-coated PA gels irrespective of elastic

moduli was also shown by Tilghman *et al.* for A549 and MDA-MB-231 cancer cells (Tilghman et al. 2010).

Cell spreading area

Most, but not all, of the cell lines showed greater cell spreading on stiffer than on softer substrates. The cell lines SW620 and PC3, for which proliferation was independent of stiffness, were also irresponsive to stiffness with respect to cell spreading. Cell lines that demonstrated rigidity-dependent growth also showed rigidity-dependent spreading, with the exception of HT29 (Figure 5, Supplemental Figure 4). Both ACHN and MDA-MB-231 cell lines exhibited >2 fold increase in mean spreading area on PA gels of 100 kPa as opposed to gels of 1 kPa, SY5Y, HepG2, HT29, and 786-0 all showed ~1.5 fold increase, while HeLa showed only ~1.2 fold increase. Our findings that cell response to stiffness differs between cell lines are in good agreement with the literature (Yeung et al. 2005). For example, Tilghman *et al.*, reported no change in cell spreading area for PC3 cells, but >2-fold increase in cell spreading area on 4.8 kPa versus 0.15 kPa collagen-coated PA gels for MDA-MB-231 cells. Further, Lam *et al.* observed >2 fold increase in average neurite length of SH-SY5Y cells on collagen-coated PA gels of 50 kPa versus 1 kPa (Lam et al. 2010).

Compared to the other cell lines, the breast cancer cell line MCF-7 exhibited a unique response. The cells formed 3D cell aggregates on the soft 1 kPa matrices and 2D cell sheets on the stiffer 10 kPa and 100 kPa matrices (Supplemental Figure 4), which prevented us from measuring cell spreading area of individual cells. Similar behavior for breast cancer cells MCF-10A cultured on 400 Pa, where they formed 3D aggregates, and 1250 Pa PA gels, where they formed 2D aggregates, was observed by Shebanova *et al.* and was attributed to decreased mobility and increased cell-cell interactions on the soft substrates (Shebanova and Hammer 2012). The spreading area of the MCF-7 cell aggregates after 72 h of culture was ~20 times smaller for the 1 kPa gel than for the 10 and 100 kPa gels, possibly due to higher proliferation on the stiffer gels as observed on Figure 3.

Screening substrate stiffness-dependent drug responses

The developed stiffness assay, due to its reproducibility, low cost, and multiwell format, is an excellent means for testing drug cytotoxicity. To demonstrate the utility of the developed multiwell assay as a drug-screening platform we tested the effect of paclitaxel on cancer cells. Cell viability was measured with an MTS assay, which compared well to the gold standard for assessing drug toxicity – sulforhodamine B (Skehan et al. 1990; Voigt 2005) (see Supplemental Figure 5). To optimize the cells' response to Paclitaxel, all drug screening tests were conducted with cells seeded at 10^4 cells/well (Dimanche-Boitrel et al. 1992) (see Supplemental Figure 6). To increase throughput, we chose two gel stiffnesses, namely 1 kPa and 100 kPa and subset of the tested cell lines.

Figure 6 shows the dose-response curves for cells that exhibited stiffness-dependent drug response, namely HeLa and SY5Y (Figure 6 a,b), and cells that seemingly exhibited stiffness-independent drug response, namely MCF-7, PC3, HepG2, 789-0, SW620, and HT29 (Figure 6 c-h). The generated dose-response curves were further used to calculate the IC_{50} for each cell type (Table 3). Our results indicate that majority of the cell lines studied

responded to paclitaxel equally well on soft or stiff gels. It has to be emphasized that some of the cell lines, such as HepG2 and 786-0, which exhibited strong proliferative dependence on substrate stiffness, exhibited a stiffness-independent response to paclitaxel. For two of the cancer cell types, namely HeLa and SY5Y, paclitaxel was more cytotoxic to the cells on gels of lower stiffness, with IC₅₀ values ~2 and 3.5 times higher on the 100 kPa gels as compared to the 1 kPa gels, respectively.

Systematic studies on the effect of substrate stiffness on the cancer cell response to cytotoxic drugs had not been performed prior to this study, so it is difficult to compare our results directly to the literature. However, stiffness-dependent drug response has been noted previously, albeit in a different context. For example, in studying the response of lung fibroblasts to different anti-proliferative drugs, Mih *et al.* observed that paclitaxel was highly effective on rigid glass but less so on soft PA gels (Mih *et al.* 2011). Schrader *et al.* observed that hepatocellular carcinoma cell lines, HepG2 and Huh7, showed decreased apoptosis on stiff (12 kPa) as opposed to soft (1 kPa) gels in response to cisplatin treatment (Schrader *et al.* 2011). In general, it has been suggested that stiffer environments may enhance cell survival in growth-restrictive circumstances by providing resistance to apoptosis (Mih *et al.* 2011; Tilghman *et al.* 2010). In another recent study on drug resistance mechanisms, Eker *et al.* (Eker *et al.* 2013) demonstrated that drug resistant MCF-7 breast cancer cells exhibited a denser fiber-like network of actin filaments, loss of tight junctions and lower levels of peripheral actin, suggesting that remodeling of the actin cytoskeleton might be the reason behind drug resistance. Interestingly, higher substrate stiffness has also been associated with a denser actin network (Yeung *et al.* 2005) and disruption of cell-cell junctions (Kim and Asthagiri 2011). Cytoskeletal re-organization on stiffer substrates has also been linked to cell stiffening up to a threshold stiffness value (Solon *et al.* 2007), while cell stiffening itself has been linked to chemotherapeutic drug resistance (Sharma *et al.* 2012). Collectively, these findings suggest that stiffer substrates would be associated with higher drug resistance, corroborating the data presented here for HeLa and SY5Y cells. However, more detailed investigations are needed to elucidate the mechanisms underlying the observed stiffness-dependent drug responses. Although such investigations are outside the scope of this study, the developed stiffness assay should facilitate similar future research on stiffness-induced mechanisms of drug resistance.

CONCLUSIONS

In conclusion, we have described the development of a multiwell stiffness assay that is suitable for cytotoxicity drug screening. The assay is an improvement over existing methods in several aspects: it is quick to manufacture, it robustly produces homogenous gels, and requires only materials that are available for purchase. We validated the assay by testing several cancer cell lines in terms of proliferative capacity, cell spreading area, and paclitaxel responsiveness as a function of stiffness. Our results demonstrated that for most cell types, proliferation and cell spreading area are increased on stiffer substrates. We also demonstrated that changing substrate stiffness alters cell response to paclitaxel in some cell types but not others. Current drug screening assays mostly are performed in rigid tissue culture plates but our finding suggests that physiological stiffness context might be more

appropriate, which has implications for building predictive biomaterial-based in-vitro assays for drug screening.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Raimon Sunyer for valuable technical discussion and practical help with performing atomic force microscopy measurements of gel stiffness, Dr. Elena Makareeva and Dr. Sergey Leikin for help with the fluorescent collagen labeling, Danielle Ferguson for technical assistance, and Colleen Cole for image analysis assistance. This work was supported by funds from the Intramural Research Program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health.

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Figure 1.

Schematic of polyacrylamide gel preparation and incorporation into a multiwell plate. The gels were formed between two GelBond flexible sheet supports, where the gel is attached to the bottom support. The top sheet is then peeled off; the gel is dried and cut into pieces that conform to the wells of a multiwell plate. The gels are glued to the bottom of the well with a drop of PDMS, then collagen-coated, and sterilized via UV irradiation.

Figure 2.

Confocal cross-sectional fluorescence images confirm that collagen (light gray) functionalization is uniform along the PA gel surface for gels of various stiffness. Red fluorescent latex beads (dark grey on image) were embedded within the gels to aid visualization. The images represent gels with Young's modulus of (a) 1 kPa, (b) 10 kPa, and (c) 100 kPa. Scale bar = 100 μm .

Figure 3.

Cell proliferation is affected by gel stiffness in a cell-dependent manner. Cell proliferation was measured by an MTS assay on 1 kPa gels (gray bars) or 100 kPa gels (dashed line). To aid comparison between different cell lines, cell proliferation was normalized by the cell proliferation on the 100 kPa gels for each cell type (signified by the dashed line); $n = 3$. Asterisks designate statistical differences from cell proliferation on the 100 kPa gels ($p < 0.05$).

Figure 4.

Cell adherence is not affected by gel stiffness. Cell adherence was measured by an MTS assay 3-5 h post cell seeding on 1 and 100 kPa gels; To aid comparison between different cell lines, cell adherence was normalized by the cell adherence on the 100 kPa gels for each cell type (signified by the dashed line); $n = 3$. No significant differences were noted between the OD measurements for gels of different stiffness ($p < 0.05$).

Figure 5.

Mean cell spreading area is affected by PA gel stiffness. All cell lines, with the exception of HT29, PC3, and SW620, exhibited significant increase in cell spreading area on 100 kPa PA gels as opposed to 1 kPa PA gels. Cell spreading area was measured 72 h post cell seeding; $n = 3$. Asterisks designate significant differences ($p < 0.05$).

Figure 6.

Cancer cell responsiveness to paclitaxel as a function of gel stiffness. In some cases, cell response is seen to be dependent on substrate stiffness: (a) Hela, (b) SY5Y. In others, cell response to paclitaxel is independent of substrate stiffness: (c) MCF-7, (d) PC3, (e) HepG2, (f) 786-0, (g) SW620, (h) HT29. All cells were seeded on 96-well stiffness assay 24 h prior to a 72 h Paclitaxel exposure. Cell viability was measured by an MTS assay. The OD was normalized as follows: (OD₄₉₀ of cells at X nM Paclitaxel - OD₄₉₀ of cells at 100 nM

Paclitaxel)/(OD₄₉₀ of cells at 0 nM Paclitaxel - OD₄₉₀ of cells at 100 nM Paclitaxel) × 100;
n = 2.

Table 1

Polyacrylamide gel composition: amount of acrylamide and bis-acrylamide used to make gels of a desired stiffness.

Gel stiffness, kPa	Acrylamide	Bis-acrylamide
1.06 ± 0.27	5%	0.025%
10.60 ± 3.10	8%	0.1%
102.52 ± 28.79	12%	0.25%

Table 2

List of cancer cell lines and tissue of origin.

Cell line	Cell line type
HepG2	Hepatocellular liver carcinoma
SY5Y	Neuroblastoma
MDA-MB-231	Mammary gland adenocarcinoma
MCF-7	Mammary gland adenocarcinoma
HeLa	Cervical adenocarcinoma
SW620	Colorectal adenocarcinoma
HT29	Colorectal adenocarcinoma
786-0	Renal cell adenocarcinoma
ACHN	Renal cell adenocarcinoma
PC3	Prostate adenocarcinoma

Table 3

Cell responsiveness to paclitaxel as a function of gel stiffness, represented as the drug IC₅₀ (nM). Values for IC₅₀ were calculated by fitting the data to a four-parameter dose-response curve as described in Methods. It appears that all cell lines tested, with the exception of HeLa and SY5Y, respond to paclitaxel equally well on soft, 1 kPa, and stiff, 100 kPa, substrate; n = 2.

Cell type	Stiffness (kPa)	IC ₅₀ *	Confidence interval	R ²
HeLa	1	2.3	1.7 - 3.2	0.94
	100	7.4	5.6 - 9.9	0.94
SY5Y	1	1.7	1.3 - 2.3	0.94
	100	6.0	5.0 - 7.3	0.97
786-0	1	38.0	29.6 - 48.8	0.93
	100	51.3	33.6 - 78.4	0.80
HepG2	1	8.3	4.4 - 15.5	0.77
	100	7.9	5.4 - 11.7	0.89
HT29	1	4.7	3.3 - 6.7	0.92
	100	2.8	1.9 - 4.2	0.90
MCF-7	1	3.2	1.9 - 5.2	0.85
	100	8.3	4.9 - 13.9	0.84
PC3	1	13.0	11.6 - 14.6	0.98
	100	12.9	9.7 - 17.1	0.91
SW620	1	3.6	2.9 - 4.5	0.94
	100	2.6	2.3 - 2.9	0.98