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Guanidine-Containing Methacrylamide (Co)polymers *via a*RAFT: Toward a Cell Penetrating Peptide Mimic[a]

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Abstract

We report the synthesis and controlled radical homo- and block copolymerization of 3-guanidinopropyl methacrylamide (GPMA) utilizing aqueous reversible addition-fragmentation chain transfer (*a*RAFT) polymerization. The resulting homopolymer and block copolymer with N-(2-hydroxypropyl) methacrylamide (HPMA) were prepared to mimic the behavior of cell penetrating peptides (CPPs) and poly(arginine) (> 6 units) which have been shown to cross cell membranes. The homopolymerization mediated by 4-cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) in aqueous buffer exhibited pseudo-first-order kinetics and linear growth of molecular weight with conversion. Retention of the "living" thiocarbonylthio ω-end-group was demonstrated through successful chain extension of the GPMA macroCTA yielding $GPMA_{37}$-b-$GPMA_{61}$ (M_w/M_n =1.05). Block copolymers of GPMA with the non-immunogenic, biocompatible HPMA were synthesized yielding $HPMA_{271}$-b-$GPMA_{13}$ (M_w/M_n = 1.15). Notably, intracellular uptake was confirmed by fluorescence microscopy, confocal laser scanning microscopy, and flow cytometry experiments after 2.5 h incubation with KB cells at 4 °C and at 37 °C utilizing FITC-labeled, GPMA-containing copolymers. The observed facility of cellular uptake and the structural control afforded by *a*RAFT polymerization suggest significant potential for these synthetic (co)polymers as drug delivery vehicles in targeted therapies.

Keywords

aqueous reversible addition-fragmentation chain transfer polymerization; aRAFT; cell-penetrating peptides; CPPs; guanidine-containing methacrylamido monomers and copolymers; water-soluble copolymers

In recent years, there has been extensive research regarding the unique cellular uptake properties of cell penetrating peptides (CPPs).[1–4] Common CPPs, such as Tat and poly(arginine), are small (< 20 nm), cationic, and can cross the plasma membrane of most mammalian cells.[5] Significantly, cell entry of both Tat and poly(arginine) oligopeptides can occur *via* an endocytotic-independent pathway, although the precise mechanism is still debated.[5–12] The enhanced cellular uptake of these peptides is reported to depend on the

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Supporting Information. Experimental details and spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

presence of basic amino acid sequences rich in arginine residues and not on peptide secondary structure.[5, 12, 13] Wender and coworkers have further demonstrated the cell penetrating properties of arginine by synthesizing a D-arginine oligomer (9 units) that exhibited >100 fold increase in cellular uptake over $Tat_{49–57}$.[12]

One of the most attractive features of CPPs is their ability to transport macromolecules easily across cellular membranes. Additionally, analysis of *in vivo* tissue samples reveals uptake into most tissues including the brain.[2, 14] Based on observed trans-membrane transport alone, modification of synthetic drug delivery vehicles with CPPs appears to hold great promise in targeted therapies. Cellular uptake of synthetic drug delivery vehicles may occur through one of several endocytotic pathways. In order for efficacious delivery, the internalized vehicle and/or the delivered cargo must escape the endosome prior to lysosomal degradation or exocytosis. Some suggested mechanisms for assisting escape involve membrane disruption (i.e. proton sponge effect, fusogenic peptides). Another potential strategy for efficacious delivery involves bypassing endocytosis altogether, a process that conceptually would avoid lysosomal degradation and/or exocytosis of the packaged therapeutic. For example, Kopecek and coworkers conjugated a Tat peptide to a biocompatible copolymer that was subsequently internalized into ovarian mammalian cancer cells through both endocytotic and non-endocytotic pathways. [15, 16] However, despite the success in cell uptake, the difficulty in synthesis and low conjugation efficiency demonstrated the need for a more direct route. Funhoff et al. polymerized a guanidine-containing methacrylate by classical free radical polymerization and condensed plasmid DNA into small polyplexes that successfully transfected COS-7 cells in the absence of serum. In the latter case, however, cellular uptake of the free (uncontrolled) polymer and its complexes was mainly endocytotic in nature rather than *via* direct cell penetration. [17] Inspired by the above work and drawing from our previous success at controlled aqueous polymerization, we targeted (co)polymer architecture that might mimic the cell-uptake behavior of Tat, poly(arginine), or other guanidine-pendent polyplexes.

Controlled polymerization techniques now allow tailored block lengths and advanced architectures while maintaining narrow molecular weight distributions. Here we report the aqueous reversible addition-fragmentation chain transfer (*a*RAFT) polymerization of guanidine-containing monomers directly in water without protecting groups, thus adding an additional synthetic pathway for highly functional systems.[18–22] Previously, RAFT polymerizations have been reported with a variety of functional monomers including anionic,[23–25] zwitterionic, [26,27] and neutral[28–40] in both organic and aqueous media. [41–48] For example, our group conducted the initial controlled polymerization of the cationic methacrylamide monomer, *N*-[3-(dimethylamino)propyl]methacrylamide (DMAPMA), using aqueous media and 4-cyanopentanoic acid dithiobenzoate (CTP) as the chain transfer agent.[18] An acidic environment was necessary in order to obtain controlled molecular weight (M_n) and low M_w/M_n values.

In order to provide a controlled synthetic mimic for cell penetration, we first prepared 3-guanidinopropyl methacrylamide (GPMA) and subsequently conducted its polymerization *via a*RAFT in an acetate buffer solution and mediated by cyano-4-(ethylsulfanylthiocarbonylsulfanyl)pentanoic acid (CEP) as the chain transfer agent. The polymerization showed linear molecular weight dependence with conversion, yielding control over both M_n and M_w/M_n. Chain extension of the polyGPMA macroCTA was successfully accomplished by adding GPMA, as was block copolymerization by adding GPMA to the poly HPMA macroCTA. HPMA was chosen as a comonomer since poly(HPMA) has the reported attributes of being biocompatible, non-immunogenic, and sufficiently hydrophilic to promote the enhanced permeability and retention (EPR) effect that allows accumulation within tumoral tissue. [49,50]

In our work we adapted a one-step approach to the synthesis of GPMA first reported by Shea et al.[51] We utilized amino propyl methacrylamide (APMA) and 2-ethyl-2-thiopseudourea hydrobromide (Scheme 1) to prepare the methacrylamide monomer in 72% yield (full experimental description and characterization (Schemes S1–S3), Figures S1–S4 can be found in the electronic supporting information). The guanidinium group, while not a strong nucleophile, is very basic with the guanidinium cation having a pka of around 13. The polymerization kinetics, shown in Figure 1, were determined using CEP as the chain transfer agent and V-501 as the initiator at two initial monomer concentrations ($[M]_0$: 0.5 and 1.0 M). The linearity of the kinetic plots (Figure 1, top) up to 97% conversion demonstrates pseudo-first order behavior for the polymerization. The refractive index traces were symmetrical and shifted to lower elution volumes as the reaction proceeded (Figure 1, bottom). As often observed for CEP-mediated RAFT polymerization of acrylamido monomers, after an early initialization period, experimental molecular weights (close to those theoretically predicted) and narrow M_w/M_n values were observed with conversion (Figure 1, middle graph and Table 1).

To further verify the livingness of the *a*RAFT polymerization of the GPMA monomer, we conducted blocking experiments with a PGPMA macro-CTA (Figure 2, top) and a PHPMA macro-CTA (Figure 2, bottom). PGPMA macro-CTA was synthesized in acetate buffer at pH 5.2 with CEP as the chain transfer agent and V-501 as the initiator. The polymerization was quenched after 6 h (17% conversion) and an aliquot was taken for GPC analysis (Figure 2, top). Additional V-501 was added and the reaction was allowed to proceed overnight (98% conversion). The shift in elution volume (Figure 2, top) of the $GPMA_{37}$-*b*-$GPMA_{61}$ homopolymer shows that the chain ends remained active with no detectable hydrolysis or aminolysis.

An HPMA-*b*-GPMA copolymer was also synthesized in order to prepare copolymers that structurally mimic CPPs or poly(arginine). The HPMA macroCTA was synthesized as previously reported.[21] Following purification via dialysis and lyophilization, the macro-CTA was chain extended with GPMA using V-501 as the initiator. Since poly(arginine) exhibits optimum cellular uptake at lower (>6 repeat units)[8, 9] segmental lengths, block copolymers consisting of a long HPMA block (X_n = 271) and a short GPMA block length (X_n = 13) were targeted. Chain extension was successful as demonstrated by the shift in elution volume (Figure 2, bottom) of the block copolymer.

The ability of $GPMA_{98}$ and $HPMA_{271}$-*b*-$GPMA_{13}$ to enter cells via both endocytotic and non-endocytotic pathways was probed by incubating the polymers with KB cells at both 37 °C and 4 °C. At 37 °C, all energy dependent endocytotic pathways (clathrin-mediated endocytosis, caveolae, macropinocytosis, and phagocytosis) are operational.[52] However, at 4 °C, ATP production is slowed considerably, and these pathways are thus inhibited.[53] Therefore, uptake of any macromolecular structure should occur via an alternative uptake mechanism. Polymers were labeled with an amine-containing FITC dye *via* the carboxylic acid end group of the polymer using EDC coupling with sulfo-NHS in acetate buffer at pH 6. The polymer samples ($HPMA_{458}$, $GPMA_{98}$, and $HPMA_{271}$-*b*-$GPMA_{13}$) were incubated with KB cells for 2.5 h at both 37 °C and 4 °C. Each polymer sample was examined using fluorescence microscopy (Figure 3), confocal scanning laser microscopy (Figure S6) and flow cytometry (Figure S7). As expected, the HPMA homopolymer did not enter cells at 37 °C or 4 °C for the short incubation time period (Panels A & B in Figure 3) since there is no moiety for direct uptake.[15, 16] Both the GPMA homopolymer and the cell penetrating peptide mimic ($HPMA_{271}$-*b*-$GPMA_{13}$) showed significant uptake into KB cells after incubating 2.5 h at 37 °C and 4 °C. In addition, flow cytometry results indicated that the $HPMA_{271}$-*b*-$GPMA_{13}$ copolymer had increased uptake compared to the GPMA homopolymer at 37 °C (Figure S7B). Fluorescence microscopy and flow cytometry of KB

cells incubated with polymer at 4 °C showed both $GPMA_{98}$ and $HPMA_{271}$-*b*-$GPMA_{13}$ entering cells (Panels D & F, Figure 3, and Figure S7B). The cell count and mean fluorescence for these cells were significantly lower than those observed for tests conducted at 37 °C (Table S1). To confirm polymer entry into the cells, cellular cross sections from top to bottom were examined by Z-stack confocal laser scanning microscopy (Figure S6). Together the above results suggest that $GPMA_{98}$ and $HPMA_{271}$-*b*-$GPMA_{13}$ may enter the cell through both endocytotic and energy-independent pathways. Although not the primary focus of this communication, further studies will be necessary to fully understand the cellular uptake behavior and capabilities of these synthetic copolymers.

In conclusion, we successfully synthesized homo- and block copolymers of a guanidinium-containing methacrylamide monomer using *a*RAFT polymerization. The block copolymer $HPMA_{271}$-*b*-$GPMA_{13}$ and homopolymer $GPMA_{98}$ were incubated with KB cells at both 37 °C and 4 °C to investigate the mechanism of uptake. Fluorescence microscopy and flow cytometry results indicate intracellular uptake *via* both endocytotic and energy-independent pathways. The ability to tailor precise architectures, the molecular weight, and molecular weight distribution directly in water opens the door for guanidinium-functional polymers to be used as pro-drugs, in gene delivery or for other applications as advanced bio-materials.[22]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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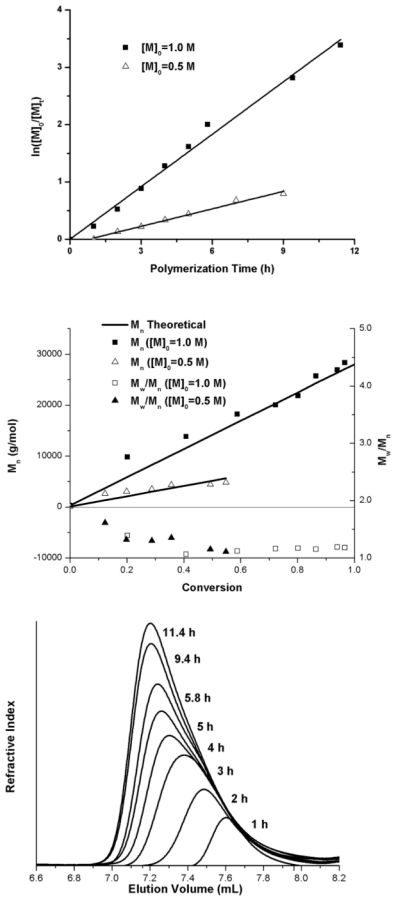

Figure 1.

Plot of $\ln([M]_0/[M]_t)$ vs. conversion of the growing GPMA homopolymer fitted to a linear prediction (top). Plot of M_n vs. conversion of the growing GPMA homopolymer with M_n theoretical and M_w/M_n(middle) and Refractive Index traces (bottom).

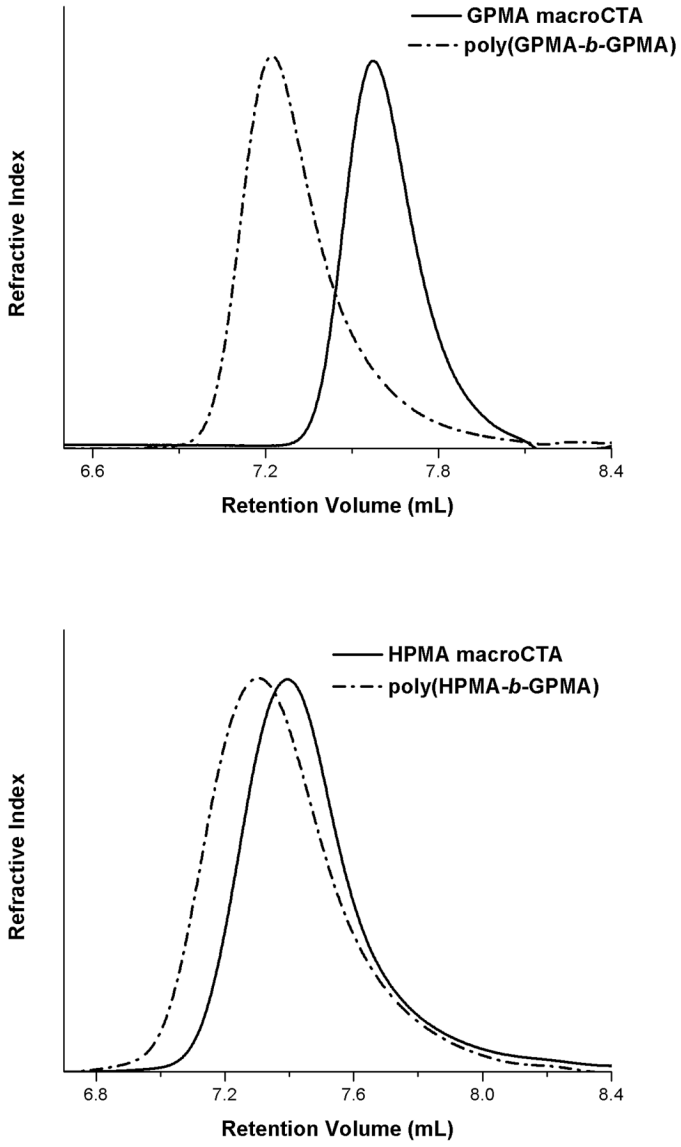

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Figure 2.

Aqueous size exclusion chromatography trace of GPMA macroCTA chain extension with GPMA to form GPMA-*b*-GPMA copolymers (top). Aqueous size exclusion chromatography trace of HPMA macroCTA chain extension with GPMA to form HPMA-*b*-GPMA copolymer (bottom).

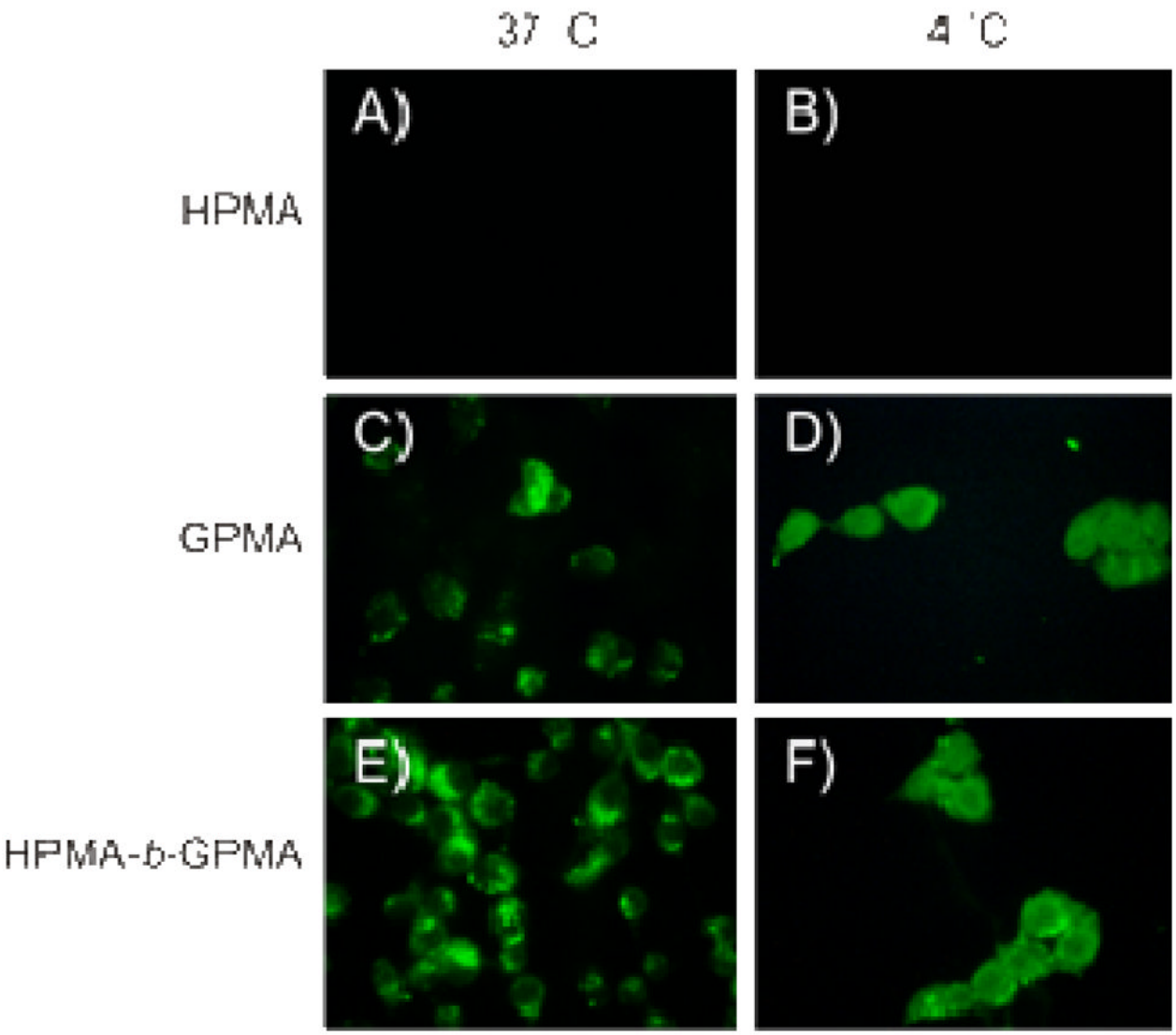
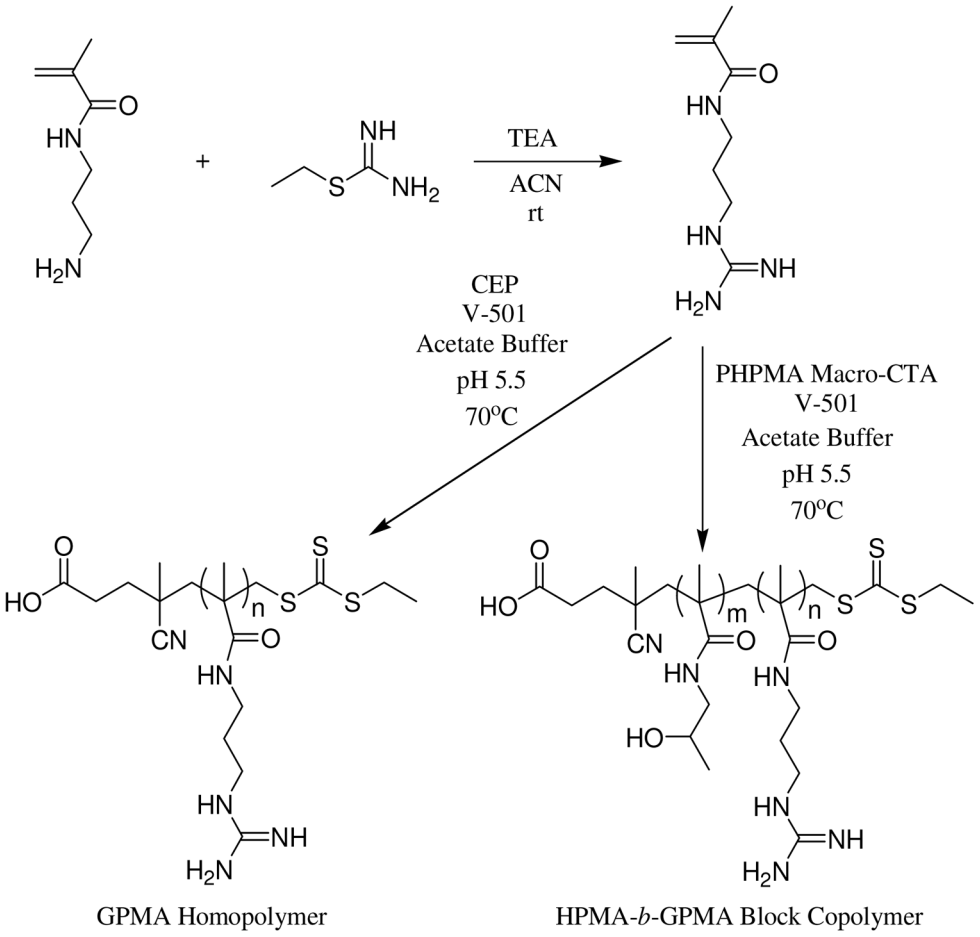


Figure 3.

Fluorescent microscopy images of FITC-labeled polymers at 37 °C (A, C, E) and 4 °C (B, D, F) in KB cells. FITC-labeled HPMA (M_n = 60,000 g/mol) is shown in A, B with no fluorescence. GPMA (M_n = 18,100 g/mol) is shown in C and D, transfecting cells at 37 °C and at 4 °C. HPMA-*b*-GPMA (M_n = 39,810 g/mol) is shown in E and F to transfect cells at both 37°C and 4 °C.



Scheme 1.
Synthesis of 3-guanidinopropyl methacrylamide (GPMA) and subsequent *a*RAFT polymerization of the monomer to form a GPMA homopolymer and HPMA block copolymer.

Table 1

GPMA homopolymers synthesized at both 0.5 and 1.0 M initial monomer concentration ($[M]_0$) in acetate buffer at 70 °C with V-501 as free radical initiator and CEP as chain transfer agent.

Time (h)	$[M]_0$	Conversion (%)	M_n (expt)	M_n (theory)	M_w/M_n
1	0.5	0	300	300	--
2	0.5	12	2800	1500	1.61
3	0.5	20	3200	2300	1.32
4	0.5	29	3700	3200	1.30
5	0.5	36	4500	3900	1.35
7	0.5	49	4600	5200	1.15
9	0.5	55	5000	5800	1.11
1	1.0	20	9800	5900	1.39
2	1.0	41	13800	11600	1.06
3	1.0	59	18200	16600	1.12
4	1.0	72	20000	20300	1.16
5	1.0	80	21800	22500	1.17
5.8	1.0	87	25700	24300	1.15
9.4	1.0	94	26900	26400	1.19
11.4	1.0	97	28300	27100	1.18